A STUDY OF THE CHANGES IN THE BLOOD AND BLOOD-FORMING ORGANS PRODUCED BY CYTOTOXIC SERA, WITH SPECIAL REFERENCE TO HÆMOLYMPHOTOXIN.[1]

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PLATES IV AND V.

Since this article was originally planned, many changes have been made in its scope and general outline, not only because the great amount of study devoted to the cytotoxins has rendered some of the work first planned unnecessary, but also because the opinions concerning the cytotoxins, their mode of action, specificity, etc., have undergone many changes. Perhaps the most radical change of ideas is in regard to specificity, many observers having bent their energies towards definitely proving or disproving the specificity of the cytotoxins. The one who has perhaps done most towards proving the non-specificity of the cytotoxins is Pearce, who, in a recent paper before the American Association of Pathologists and Bacteriologists, has clearly demonstrated that the changes which have been regarded as specific by most writers are due to hæmolysins and agglutinins; when these factors, the former in particular, are considered, the changes cannot be regarded as specific,—they are not changes in kind, but are rather changes in degree alone.

In the investigations here reported, an attempt is made to compare several cytotoxins to find what changes they produce, and, if the changes are merely in degree, to determine to what extent these changes occur after the injection of different cytotoxins, and the order in which the degree of the change would

[1] Aided by a grant from the Rockefeller Institute for Medical Research.

place them. Furthermore, an attempt is here made to produce a hæmolymphocytotoxin and by its aid to throw if possible some light upon the physiological action of the hæmolymph glands. So far no attempt has been made to produce a hæmolymphocytotoxin and to study the effects produced by it; in doing so an entirely new field is entered.

Taken for granted that the hæmolymph nodes are organs *sui generis*, as demonstrated by Warthin (1), what then is their true function? Some of the theories may be stated as follows:

1. They are hæmolytic, and therefore destructive organs, and rid the body of old and partially broken-down red blood cells.

2. They are centres for the formation of leucocytes and blood plasma, and in these respects conservative.

3. They are centres for the production of large numbers of eosinophile cells; the granules arising from the disintegration products of red cells are taken up by mononuclear leucocytes, the nuclei of which become polymorphous.

4. They compensate in some degree for the spleen in case of inability of that organ to perform its proper function.

5. They are centres for the destruction of leucocytes.

6. Since they are enlarged in certain infections, they play a part in the production of immunity and in the protection of the body through the formation of anti-bodies.

The suggestion by Warthin and Lewis (2) of a hæmolytic ferment produced by these glands brings forth more strongly the possibility that they play an important part in the production of certain forms of anæmia. In apparent confirmation of this suggestion, Warthin, in eight autopsies on cases of pernicious anæmia, has found the hæmolymph nodes exceedingly red and hyperplastic and showing signs of over-activity. One object of this paper, then, is to prove if possible the presence of such a ferment; for, if it is present, a more hæmolytic serum should be produced by the injection of hæmolymph nodes than would be produced by injection of defibrinated blood, diluted to a corresponding percentage of red blood cells.

Throughout this work it will be frequently necessary to refer to the effects of lymphotoxin and myelotoxin on the blood and

blood-forming organs. The literature of these subjects has of late become so extensive that a review of it here is impossible; some of its aspects, however, have been most excellently reviewed in the articles by Flexner (3) and by Bunting (4) to whose work references will often be made.

METHODS AND MATERIALS.

The basis of the present investigation is that a specific toxin for a tissue may be formed by the injection of that tissue into an intermediary animal. Glands were obtained from sheep and injected into geese; the serum of the goose, after a sufficient time had elapsed for attaining its highest toxicity, was injected into a sheep, the tissues of which were then examined for evidences of a specific toxin.

In producing the toxin geese and sheep were used, the former on account of their ability to withstand repeated injections without becoming infected, and the latter because the hæmolymph glands obtained from them are larger and more numerous than from the animals ordinarily used for experimental purposes.

The geese received suspensions of glands prepared by the method in general use. Glands removed under as nearly sterile precautions as possible were first triturated in a sterile mortar, suspended in sterile physiological salt solution, and allowed to extract for twelve hours; they were strained through sterile gauze and injected into geese. The injections were made intraperitoneally at intervals of from three to ten days until seven or eight injections had been made; at the end of this time the geese were bled.

When the geese were injected with blood, blood was first defibrinated and then diluted with physiological salt solution in such quantity that the percentage of red blood cells was the same as that contained in a corresponding injection of hæmolymph nodes, so that the results might be comparable.

The geese used were as follows:
Goose 2 furnished normal serum.
Goose 5 furnished normal serum.

Goose 3, 4, 6, and 12 each received seven injections of hæmolymph glands, extending over a period of about thirty-six days.

Goose 7 and 14 received seven injections of defibrinated blood diluted as above.

Goose 11 received 6 injections of lymph glands.

Goose 15 received 8 injections of endothelium.

Goose 16 received 5 injections of liver.

Goose 17 received 5 injections of kidney.

Goose 18 received 5 injections of spleen.

(Note.—Goose 1 and Sheep 1 were used in a preliminary experiment performed a year ago, but not included in the present series of experiments.)

NORMAL HISTOLOGY OF THE SHEEP.

The tissues to be examined were removed from normal sheep, fixed in formalin and mercuric chloride, and stained with hæmotoxylin and eosin. These methods were uniformly applied to the pathological organs. Various special stains were also employed when deemed necessary. For the iron reaction, potassium ferrocyanide and hydrochloric acid were used.

Blood.—Counts of the total number of leucocytes as well as differential counts were made shortly before injection of the sheep and at varying intervals for four or five days after. At the end of this time the sheep were killed and sections made from all the organs. In counting the cells of the blood, the Thoma-Zeiss apparatus was used and at the same time smears were made for differential counts, the latter being fixed in alcohol and ether and stained with methylene blue and eosin.

The average leucocyte count of a sheep is about 8000, but varies between the wide limits of from 6000 to 12,000 per cubic millimetre. The erythrocyte count may also vary greatly and still be within normal limits, but averages eight million per cubic millimetre.

In order that the results might be comparable, the following cells were taken into consideration in making the differential counts, this classification of leucocytes being used by Bunting in his work on the blood of the rabbit:

1.—Mononuclear Leucocytes,
  a—Lymphocytes,
  b—Large Mononuclears,

2.—Polymorphonuclear Leucocytes,
a—Pseudo-eosinophiles (Amphophiles),
b—Eosinophiles (coarsely granular),
c—Basophiles (Mast Cells).

The last are so few in numbers in the sheep that they might well be left entirely out of consideration. The average percentage and limits of variation as obtained from about twenty counts from normal sheep may be given as follows:

Lymphocytes.....	53 %	40 to 60 %
Large Mononuclears....	8 %	3 to 11 %
Amphophiles.....	37 %	30 to 55 %
Eosinophiles.....	1.7 %	.2 to 8 %
Mast Cells.....	.3 %	0 to 2 %

It is readily seen from the above that the different forms may vary within rather wide limits and still be normal.

Hæmolymph Glands.—The hæmolymph glands of the sheep are typical in structure, the majority being of the splenolymph type. They have a thick capsule from which trabeculæ, forming either a delicate framework or a coarse framework densely infiltrated with lymphoid tissue, extend into the gland, dividing it into irregular lobules. The lymphadenoid tissue, while varying greatly in amount, is usually abundant and forms the greater part of the gland. As a rule, large numbers of nodes with germ centres showing fairly active proliferation are present, although in a few cases cords alone are found. The peripheral blood sinus is generally large but may be frequently interrupted by lymphadenoid tissue projecting into it. The gland shows a few phagocytes, some pyknotic and vesicular nuclei, and a light sprinkling of eosinophiles, the latter being for the most part polymorphonuclear, although a few mononuclear forms are to be found.

Lymph Glands.—The lymph glands are almost identical with human glands in structure, having a thick cortical substance composed of lymph nodules and germ centres, and a medullary substance composed of anastamosing cords separated by trabeculæ. The entire gland is surrounded by a rather thick capsule.

Only a few eosinophile cells are seen and these are chiefly around the borders of the lymph nodes.

Spleen.—The spleen has a thick capsule, and the trabeculæ throughout the organ are conspicuous. The Malpighian bodies are large, distinct, and not very numerous. The lymphoid cells in the pulp are scanty, the reticulum being relatively increased, and the reticular cells large; the organ seems to show an over-abundance of connective tissue.

Bone Marrow.—The bone marrow cells found in the sheep having practically the same characteristics as those of the rabbit, the following classification as given by Muir (5) for the latter animal may here be used:

1.—Leucocytic class of cells:

a—Cells with finely granular protoplasm which constitute the great majority of the cells in the marrow. After mitotic division they become the finely granular amphophile leucocytes of the blood.

b—Cells with coarse eosinophile granules which later become the ordinary eosinophiles of the blood.

c—Hyaline or non-granular cells.

Small lymphoid cells.

Large lymphoid cells.

Large mononuclear cells.

2.—Erythrocytes.

3.—Erythroblasts:

a—Megaloblasts or large nucleated red corpuscles faintly tinted with hæmoglobin; these are the youngest variety of erythroblasts.

b—Normoblasts or typical nucleated red corpuscles, which are somewhat larger than red blood cells and are the oldest variety of erythroblasts.

4.—Giant Cells.

5.—Connective-tissue class of cells, fat cells, etc.

EXPERIMENT WITH PHYSIOLOGICAL SALT SOLUTION.

As noted by Besredka (6), Ricketts (7), Brinckerhoff and Tyzzer (8), and later by Bunting, injections of fluids intra-

peritoneally cause first a leucopenia and later a hyperleucocytosis of the peritoneal fluid. It is probable that within certain limits the greater the toxicity of the fluid injected the greater the resulting hyperleucocytosis. (Ricketts.) The bone marrow and lymph glands in attempting to compensate for this withdrawal of leucocytes from the circulation give rise to a rather typical blood picture. Bunting found after the injection of cold sterile physiological salt solution a slight rise in the total leucocyte count of the circulating blood. On differential analysis this increase was found to be due to a marked amphophile leucocytosis, at the same time that there was a correspondingly great lymphopenia. The preliminary leucocytosis he calls the "anticipatory" leucocytosis, and is due to the reaction of the bone marrow to the withdrawal of leucocytes from the circulation, the lymphocytes having a slower amœboid movement failing to respond so quickly.

As these experiments were performed on rabbits it was thought best to repeat them with sheep so that the influence of salt solution might be exactly determined for that animal and could then be eliminated from the final results. After the injection of thirty-five cubic centimetres of cold sterile physiological salt solution (Table I),* a rather sharp leucocytosis and a return to normal within twenty-four hours is obtained, and is followed by another lesser rise and fall in the next forty-eight hours. By differential counts we find at first a typical "anticipatory" leucocytosis with its accompanying lymphopenia, the lymphocytes, however, recovering quite rapidly. The second leucocytosis is due to an increase both of the amphophiles and of the lymphocytes with a final rather gradual return of both to normal.

The bone marrow, then, in the sheep as well as in the rabbit, reacts quickly to a demand upon it, the lymphocytes, because of their slower amœboid movement, responding more slowly.

INJECTION OF NORMAL SERUM OF THE GOOSE.

In the following experiments no consideration has been taken of the amount of hæmolysis *in vitro*, this being reserved for a

* The tables are given at the end of the article.

future study. The blood curve (Table II) resulting from the injection of normal serum is almost identical with that obtained by Bunting, showing a marked leucocytosis at the end of eighteen hours, followed by a fall, preceding a second and finally a third slighter rise. On differential analysis the first rise is found to be due almost entirely to an amphophile leucocytosis, except for a sharp rise of lymphocytes, with a sharper return to normal, occurring within eight hours. This preliminary increase in lymphocytes is in marked contrast, however, to the observations of Bunting, who found a typical "anticipatory" leucocytosis, as described, with its accompanying lymphopenia. The second and third rises are due entirely to an increase in lymphocytes; the number of amphophiles decrease and subsequently there is only a slight secondary rise to normal.

The large mononuclear leucocytes increase in numbers following roughly the rise and fall of the lymphocytes; the eosinophiles, after a preliminary sharp decrease in numbers, gradually rise considerably above normal and then decrease; the erythrocytes show a slight increase at first and subsequently their number remains elevated; the basophiles are so few in the sheep that they have not been enumerated in the table.

SHEEP 2.—Received 35 cc. of serum from Goose 5 at 3 P.M., December 1, 1903. A record of the leucocyte counts and differential analysis is given in Table II. The animal was killed December 8, 10 A.M. *Autopsy.*—There is slight œdema near the site of inoculation. A large amount of fluid is present in the peritoneal cavity. The hæmolymph glands are exceedingly large and numerous. All the organs and tissue are apparently normal. There is no infection.

Histology.—The structures examined showed little or no changes other than a slight hyperplasia. This is perhaps most noticeable in the bone marrow, as here the actual amount of cellular marrow is increased, the increase corresponding to a diminution in the number of fat cells. The change is due to an increase in number of the marrow cells or myelocytes. The megaloblasts and normoblasts are also more numerous than usual and show mitotic figures. The germ centres in the spleen and lymph glands stand out distinctly and show active although not excessive proliferation. Few, if any, degenerative changes are present.

The injection of normal serum of a goose into a sheep causes first a diminution in the number of the leucocytes in the circulating blood as a result of their withdrawal into the peritoneal

cavity. This fall is followed by a marked rise, due to proliferation of the antecedents of the leucocytes, first and most markedly in the bone marrow and later in the lymph glands, spleen, and hæmolymph glands. At the same time there is a slight increase in number of erythrocytes in the blood due to a proliferation of the erythroblasts in the bone marrow. The changes produced are chiefly hyperplastic, as few, if any, degenerative changes are apparent.

EXPERIMENTS WITH HÆMOLYTIC SERUM.

Hæmolytic serum was prepared by injecting a goose with normal defibrinated sheep's blood diluted with normal salt solution until it contained the same percentage of erythrocytes that is present in a corresponding injection of hæmolymph nodes.

The leucocyte curve (Table III) in this case is not very characteristic, consisting of a preliminary leucocytosis of about 2000 per cubic millimetre, followed by a still smaller increase and then a gradual rise covering a period of about seventy hours. By differential counts we find a typical "anticipatory" leucocytosis with its accompanying lymphopenia, after which there is a decrease in amphophiles and an increase in lymphocytes; the last two leucocytoses are due to the increase of lymphocytes.

The eosinophiles, after a preliminary decrease in number, gradually increase considerably above normal and then, as if a crisis had been reached, suddenly return to normal, at the end of about ninety-six hours.

The erythrocytes, after a rapid preliminary rise of one million, drop in twelve hours about two million and gradually decrease in the next thirty-two hours a million more, making a total destruction of about three million erythrocytes per cubic millimetre, or 28+% of the entire number of corpuscles.

SHEEP 9.—Received intraperitoneally 35 cc. of serum from Goose 7 at 11.30 A.M., February 9, 1904. At 3 P.M. the sheep appeared sick. The blood is dark in color; at 10.30 P.M. the blood is light in color and watery. February 11, at 5 P.M., one normoblast is found in a differential count of 500 cells. February 12, 9 A.M., two normoblasts are seen. February 14, 9 A.M., one normoblast is found. The sheep was killed February 15, 11.30 A.M. *Autopsy.*—There is

no excess of peritoneal fluid. The hæmolymph glands are dark in color. The bone marrow is light in color. Axillary, mesenteric, and retroperitoneal lymph glands are dark in color. All other organs and tissues are apparently normal. There is no infection.

Histology.—The hæmolymph glands show few changes resulting from the injection of hæmolytic serum. The glands are somewhat congested and show evidences of increased erythrocyte destruction; large endothelial phagocytes are filled with pigment and partially broken-down corpuscles. Nuclear destruction in the lymphoid tissue is perhaps somewhat more abundant than normal, and large vesicular and in some cases pyknotic nuclei are present. The eosinophiles are increased in number.

The lymph glands show distinct evidences of proliferation in the germ centres, which are large and well developed. Evidences of erythrocyte destruction are much more marked than under normal conditions. Numerous large brown pigment granules are present, for the most part within phagocytes. Numerous vesicular and pyknotic nuclei and phagocytes containing broken nuclei are also present. There is some increase in eosinophiles.

The bone marrow shows an increase of mature amphophiles together with rather marked increase of the myelocytes. There is also a distinct increase of megaloblasts and normoblasts. In all of the tissues examined there are rather marked proliferative changes in the endothelium.

The injection of a hæmolytic serum into a sheep not only causes an increased destruction of erythrocytes, but has an effect on the leucocytes and indirectly at least on the leucocyte-forming organs. Although the changes in these organs are not pronounced, and in this case, at least, not very characteristic, they are nevertheless evident. The increase in both lymphatic and hæmolymphatic glands of endothelial phagocytes containing red cells and pigment is the most striking histological change found. In connection with these evidences of hæmolysis within cells is to be noted the increase of eosinophiles.

EXPERIMENTS WITH HÆMOLYMPHOCYTOTOXIC SERUM.

In two instances sheep were injected with hæmolymphocytotoxic serum. The first gave a more typical alteration of the leucocytes, but the animal was killed shortly after injection, in order to obtain tissues at an early stage; in the second animal the leucocytes were allowed to return to normal.

In these experiments changes undergone by the leucocytes (Tables IV and V) have resembled those which follow the injection of normal serum of the goose, but the increase which first

occurs takes place in a shorter length of time, as if the demand has been more urgent and the tendency to proliferate given a sharper impetus. After the first rise there is a complete return to normal, followed by a second slower and slighter leucocytosis, which in turn, after twenty-four hours, is followed by a third rise.

On differential counts a typical "anticipatory" leucocytosis with its accompanying lymphopenia is observed. The first rise already described is due entirely to an amphophile leucocytosis; the second, however, is due to a lymphocytosis, and the third to a combined increase of both amphophiles and lymphocytes. It is readily seen that, with the exception of an "anticipatory" leucocytosis, these changes are almost identical with those produced by the injection of normal serum of the goose, until the third rise is reached. In the case of the normal serum this is due entirely to a lymphocytosis, while with the hæmolymphotoxic serum it is due to a combined rise of lymphocytes and amphophiles.

The large mononuclears remain practically unchanged throughout the experiment. It is noteworthy that in every case cited the eosinophiles, after a preliminary decrease, gradually reach a number considerably above normal and then, as by a crisis occurring at the end of about ninety-six hours, suddenly return to normal.

There is a marked difference between the erythrocyte curves in the two experiments. In the first there is a slight rise occurring within the first four hours and then a decided drop. Great emphasis is to be laid on this marked decrease in number of erythrocytes, their number dropping from 9,215,000 to 4,824,000 in four hours—a fall of 47 +% of entire number of corpuscles. In the second curve, however, there is a preliminary increase followed by a slight decrease, another rise, and then a decided drop from 15,760,000 to 13,400,000 in twelve hours, there being altogether a destruction of 2,360,000 corpuscles, or about 15% of the entire number present. In both instances after this marked decrease in number there is a gradual rise to normal, and in both instances this regeneration is somewhat excessive, the erythrocyte count exceeding normal.

SHEEP 3.—Received 35 cc. serum from Goose 3 at 2 P.M., November 6, 1903. At the time of the count on November 6, at 10.30 P.M., the blood was noticeably lighter in color and more watery than normal. The animal was killed November 10, at 10 A.M. *Autopsy* showed slight œdema around the site of inoculation. A large amount of fluid was present in the peritoneal cavity. All of the lymph glands, with the exception of the hæmolymph glands, were large, hard, and extremely dark in color.

The spleen is small, being not over two-thirds its normal size. One kidney is markedly atrophic, the other showing compensatory hypertrophy. On section the pelvis is large and dilated; the cortex is thin. The liver is large, dark in color, and bleeds freely on section. The bone marrow is normal in appearance. There is no infection.

Histology.—The hæmolymph glands show a disappearance, or at least a diminution, in size and numbers of the distinct proliferating germ centres found in normal glands. In twenty-five glands examined exceedingly few germ centres are found, and those present are small and show few mitotic figures. The blood sinuses are somewhat congested and contain large numbers of leucocytes. Eosinophile cells (Fig. 2) are present in the lymphoid tissue in large numbers; most of them are polymorphonuclear, but some are mononuclear, and a few even polynuclear. There is less evidence of cell destruction in the hæmolymph glands than might be expected, only a few large vacuolated and some pyknotic nuclei being seen. Abundant endothelial proliferation is found, and large phagocytes containing broken-down erythrocytes and blood pigment are present in great numbers.

The germ centres in the lymph glands, while more numerous than in the hæmolymph glands, are small and do not show active proliferation. There is, however, an enormous amount of proliferation in the endothelium. The amount of blood pigment in some of the lymph glands is enormous, giving the glands on macroscopical examination a dark brown or blackish color. The pigment on microscopical examination (Fig. 3) is found in the medullary portion of the gland and is chiefly intracellular, having been taken up by phagocytes. It does not contain iron, and is probably hæmatoidin formed as the result of erythrocyte destruction. Other evidences of cell destruction are not more frequently observed than in the hæmolymph glands. There is considerable increase in the number of eosinophiles present.

In the spleen, as in the other tissues examined, the germ centres are small and are not actively proliferating. The spleen is very markedly atrophic, but this atrophy must have antedated the time of injection. Some pigment is present which, in sharp contrast to that found in the lymph glands, is iron-containing and chiefly extracellular.

The bone marrow shows a marked diminution in the number of mature cells, i. e., cells ready to enter the circulation. The marrow cells, with few exceptions, however, are in some stage of mitotic division. Megaloblasts and normoblasts are also increased in numbers, the former showing active proliferative changes. The endothelium is proliferating actively. Eosinophiles are decreased in numbers. A few large vacuolated nuclei are to be seen.

SHEEP 6.—Received 35 cc. serum from Goose 6 at 1 P.M., January 22, 1904. There was evidence of hæmoglobinuria at the end of forty-eight hours. The

animal was killed January 29, 10 A.M.; at autopsy the organs were apparently normal.

Histology.—Since the sheep was killed seven days after injection, the tissues may be expected to show either a greater attempt at repair or a later stage of the intoxication.

The hæmolymph glands in this experiment, as in the last, show a decided diminution in size and numbers of germ centres. Evidences of cell destruction here, however, are very striking. With very few exceptions every nucleus, though not necrotic, is in some stage of fragmentation. Pyknotic, large vesicular, and vacuolated nuclei are in great abundance. Numerous phagocytes containing particles of broken-up nuclei are present. The pigment, phagocytes, and eosinophiles have the same characters as in Sheep 3.

The lymph glands show the changes described for Sheep 3, except that there is more evidence of nuclear destruction; there are vacuolated, vesicular, and pyknotic nuclei, and endothelial phagocytes are found. Blood pigment is present in considerable amount, but not to the same extent as in Sheep 3, although the phagocytes are as numerous.

The spleen in this case shows more marked nuclear destruction than in Sheep 3. A much larger amount of pigment (Fig.4) is, however, present here; it is iron-containing, and is probably hæmosiderin.

The red bone marrow is not abundant. Proliferative changes are confined chiefly to the megaloblasts. The myelocytes are not numerous, and those present do not show very active mitosis. Fully developed amphophiles are, however, numerous. Eosinophile cells are decreased in number.

After the injection of hæmolymphotoxic serum there is, as with the injection of normal serum from the goose, a sharp demand on the leucocyte-forming organs, which is responded to first by the bone marrow and later by the lymphocyte-forming organs. The bone marrow is able, in some degree, to compensate for this loss by proliferation, but the lymphocytes in the blood seem to be solely supplied by preformed cells. Histological examination seems to show that the tendency to proliferation of the cells antecedent to the lymphocytes is inhibited. This inhibitory or toxic action, while slight at first, becomes exceedingly destructive, as shown by the marked signs of degeneration in the lymphadenoid tissue.

The differences in changes affecting the erythrocytes in the two experiments may be partly due to the pathological condition of the kidneys in Sheep 3. In both experiments there has been a preliminary rise in the number of erythrocytes, then a fall, and finally a slow recovery which becomes excessive and carries the erythrocyte curve above normal. In Sheep 3, the

kidneys being pathological, it is probable that the excretion of the toxin has not been carried on to the same extent as in Sheep 6. Since this animal has been able to excrete some part of the toxin, the effect has occurred slowly and is less marked.

Evidence of erythrocyte destruction in the tissues is somewhat more marked in Sheep 3 than in Sheep 6, but this difference again is partly referable to the kidneys of the former; for in the latter hæmoglobinuria was present, while in Sheep 3 there was no evidence of it. The regeneration of erythrocytes is explained by the marked proliferation of megaloblasts in the bone marrow.

PARALLEL EXPERIMENTS WITH HÆMOLYMPHOTOXIN AND WITH HÆMOLYSIN.

Control experiments were here tried with hæmolymphotoxic and erythrolytic serums with the special purpose of determining the amount of hæmolysis in either case and of making careful comparisons.

To this end for corresponding experiments geese and sheep were selected respectively as nearly alike as possible; they had been kept under similar conditions. The geese were injected with the same amount of fluid in each case, and each injection contained the same amount of blood as determined by Gower's hæmoglobinometer; the goose injected with emulsion of hæmolymph gland received the same number of erythrocytes as the one receiving the blood. The conditions of the experiments were made parallel in every respect, so that the results obtained might be exactly compared.

I. *Experiment with Hæmolymphotoxin.*—Changes in the number of leucocytes (Table VI) obtained by injecting hæmolymphotoxin from the goose selected resemble in general those of Table IV, obtained after injection of hæmolymphotoxin from Goose 3; twice their number rises markedly. Examination of differential counts shows that the preliminary leucocytosis in both cases is due at first to an amphophile leucocytosis, later aided by a lymphocytosis. The second rise, however, is markedly different in the two cases, for in one instance it is

entirely due to an amphophile leucocytosis, while in the other it is wholly a lymphocytosis.

The erythrocytes in Sheep 12 after a preliminary rapid rise gradually but steadily decrease for fifty-six hours and then as gradually return to normal. Altogether there is a drop from 4,800,000 to 3,240,000, or a total destruction of 32% of the entire number of erythrocytes present.

The eosinophiles show the usual preliminary decrease, the gradual rise, and the so-called crisis.

SHEEP 12.—Received 40 cc. serum from Goose 12 at 1 P.M., April 23, 1904. Killed April 28, 4 P.M. *Autopsy.*—Hæmolymph glands are not numerous, but are large; lymph glands are rather dark in color. The liver has a peculiar greenish tinge. The bone marrow is anæmic. Otherwise the organs are normal and there is no infection.

Histology.—In sharp contrast to the other instance in which hæmolymphocytotoxin has been injected, this one shows the germ centres in the hæmolymph glands to be large, numerous, and actively proliferating. In other respects, however, they resemble those already described, in that there are a large number of degenerative changes, marked proliferation of endothelium, many phagocytes containing blood pigment and corpuscles, and a marked increase of eosinophiles.

In the lymph glands again the germ centres are large, numerous, and actively proliferating; otherwise they resemble those heretofore described. The amount of blood pigment and the number of phagocytes present in some of the glands is very large.

The spleen resembles those previously described very closely, except that there is a very little blood pigment present. Part of this pigment is hæmotoidin and is intracellular, while a part is hæmosiderin, and, situated chiefly outside the cells, is present in large amount.

The bone marrow is the seat of active proliferation and contains large numbers of mature cells, i. e., fully developed amphophiles.

II. *Experiment with Hæmolysin.*—In the sheep which received hæmolytic serum from Goose 14, as in the other experiment with hæmolytic serum, the leucocytes do not show very characteristic changes (Table VII); their number undergoes two slight but rather prolonged rises and falls. These changes are almost wholly referable to the amphophiles, the lymphocytes varying only slightly throughout the experiment.

The erythrocytes decrease slightly at first and then gradually rise to a number somewhat above normal. This number then suddenly drops from 5,340,000 to 4,280,000 in twelve hours,

1,060,000 erythrocytes, or 19% of the entire number in circulation, being destroyed.

SHEEP 14.—Received 40 cc. serum from Goose 14, on April 23, 1904, at 2 P.M. No erythroblasts were found in any of the preparations of blood. The animal was killed April 28, 3 P.M. At autopsy a large amount of peritoneal fluid is found. Hæmolymph glands are not numerous but are large. The spleen is slightly smaller than normal. The lungs are anæmic. Otherwise the organs are normal; there is no infection.

Histology.—The histological changes resemble very closely those already found after the injection of hæmolytic serum.

The two preceding experiments vary but little from other similar experiments already described, except that heretofore the effect of the hæmolymphotoxin has been to inhibit proliferation in the germ centres; in Sheep 12 proliferation is very active.

Under the same conditions, the serum obtained by the injection of emulsion of hæmolymph glands is more hæmolytic than that obtained by the injection of a corresponding amount of erythrocytes; in the first case there is a destruction of 32% of the entire number of erythrocytes in circulation, while in the latter there is a destruction of only 19%.

EXPERIMENTS WITH HEATED HÆMOLYMPHOCYTOTOXIN.

In the following experiment hæmolymphocytotoxin was heated to 56° C. for half an hour to destroy the hæmolytic action before injection.

Changes in blood (Table VIII) obtained in this experiment are strikingly like those obtained by Bunting after the injection of lymphotoxin. There is first a leucocytosis of moderate extent due to a combined increase of amphophiles and lymphocytes. Following this there is a still greater leucocytosis and finally a more moderate one. All three leucocytoses are due to a combined rise of both amphophiles and lymphocytes. They differ from those of other experiments by the sharp rise and fall of the lymphocytes which ordinarily rise slowly to a certain height and then gradually decline.

The erythrocytes show, at first, a slight decrease, due to the

fact that the hæmolytic action of the serum was not completely destroyed, but they soon regain their normal height, which they retain throughout the experiment. The large mononuclears vary little; the eosinophile cells show the ordinary preliminary increase and crisis as described above.

SHEEP 4.—Received 30 cc. of serum from Goose 4. at 3 P.M., November 18, 1903. This serum had, previous to injection, been heated to 56° C. for half an hour. The animal was killed 10 A.M., November 22. At autopsy slight œdema is found around the place of inoculation. There is a large amount of peritoneal fluid. The hæmolymph glands are dark in color and apparently not so large nor so numerous as normal. The spleen on section is dark in color, but is otherwise normal. All other organs and tissue are apparently normal; there is no infection.

Histology.—The hæmolymph glands are congested, the sinuses being large and densely crowded with erythrocytes. Germ centres are few in numbers, and when found are small but show fairly active proliferation. Evidences of cell destruction are not marked, although a few pyknotic and vesicular nuclei are to be found. Some blood pigment, probably hæmatoidin, is present. Eosinophile cells are present in great numbers and are both of mononuclear and polynuclear type, but are fewer than in Sheep 3.

Some of the lymph glands, particularly the mesenteric glands, show toxic changes, manifested by more or less wedge-shaped areas of necrosis (Fig. 1) extending into the lymph glands with their bases toward the periphery. These areas appear as granular eosin-staining masses around which in some cases there is a beginning attempt at sequestration by leucocytes. Phagocytes containing fragmented nuclei are very abundant. The germ centres, while not large, show fairly active proliferative changes. Some blood pigment, probably hæmatoidin, is present, and is contained entirely in large phagocytes. There is an enormous increase in eosinophiles, both mononuclear and polynuclear.

The spleen is congested and shows signs of degeneration, pyknotic and fragmented nuclei being present. There is a large increase of eosinophiles. A small amount of blood pigment is present.

The changes in the bone marrow consist chiefly in a marked increase in number of the myelocytes. Mature amphophiles are also numerous, eosinophiles are decreased in numbers, and lymphocytes are numerous. Throughout all the tissues examined there is a marked proliferation of endothelium.

The kidneys in this animal, in contrast to the others cited, show the presence of iron situated in the tubules and demonstrable by the appropriate reaction.

Histological examination shows that the amphophile leucocytosis is due to the proliferation of cells in the bone marrow antecedent to the amphophiles, while the increase of lymphocytes is due almost entirely to the existence of preformed cells, since proliferation in the germ centres is not marked.

A second experiment was conducted in exactly the same way as the preceding; the serum of a goose which had been immunized to hæmolymph glands being heated to 56° C. for a period of half an hour. It was expected that at the expiration of this time the hæmolytic property, at least that due to immunization with injected erythrocytes, would be destroyed.

Changes in the blood (Table IX) following the injection of this serum into the sheep resemble in only one point those obtained in the preceding experiment; there is at first leucopenia followed by a slight, gradual leucocytosis, after which there is a slight drop, another slight rise, and then a gradual return to normal. Changes affecting the entire number of leucocytes are closely followed by those of the lymphocytes, the amphophiles being so few in numbers that they have little effect upon these changes.

The erythrocytes undergo a slight preliminary rise of 700,000 and then a sharp fall of two million, after which they gradually return to normal. The eosinophiles slowly but markedly increase until the sixty-fourth hour, when the so-called crisis occurs. The large mononucleus and mast cells show no noteworthy alteration.

SHEEP 19.—Received 45 cc. of serum from Goose 19 on May 16, 1904, at 1 P.M. The serum previous to injection had been heated to 56° C. for half an hour. Sheep 19 was killed at 10 A.M., May 21. There was a moderate amount of peritoneal fluid. The hæmolymph glands were rather large and numerous. All the organs were apparently normal. Bone marrow was light in color. There was no infection.

Histology.—The hæmolymph glands are congested, the sinuses being large and densely crowded with erythrocytes. The germ centres are large and actively proliferating. There is, moreover, a slight general hyperplasia of lymphoid cells. There is little evidence of degeneration. Endothelial proliferation is marked. Large numbers of phagocytes containing entire corpuscles and some containing blood pigment, hæmatoidin, are to be seen. There is an enormous increase of eosinophiles.

The germ centres in the lymph glands are also large and actively proliferating.

In this case no necrotic areas, similar to those observed in the previous experiment, are found. Degenerative changes are fairly numerous, vacuolar, vesicular, and some pyknotic nuclei being observed.

Large numbers of phagocytes containing blood pigment, hæmatoidin, are to be seen. Eosinophiles are increased in numbers. The spleen is normal,

except for the presence of a large amount of blood pigment which, situated outside of the cells, is for the most part hæmosiderin. Mitosis in the germ centres is active, and there is a rather marked proliferation of the endothelium. Eosinophiles are somewhat increased.

In contrast to the previous experiment, the kidney tubules do not show the presence of iron.

The bone marrow seems to be little affected, and is practically normal. There is, however, some increase of erythrocytes and of eosinophiles. The presence of large numbers of mature amphophiles is noteworthy when it is recalled that few were present in the circulation.

This experiment differs markedly from the one preceding it, the changes not only in the blood but in the tissues being different.

These experiments suggest, in accordance with the statement of Pearce, that necrosis is not a specific action of the serum, but is due to its erythrolytic and agglutinative properties.

The marked hæmolysis which occurred in the last experiment is, however, of great importance, for it tends to show the presence of some hæmolytic factor, which is resistant to heat and not due to immunization with erythrocytes alone.

EXPERIMENTS WITH HETEROHÆMOLYMPHOTOXIN.

In the following experiment a hæmolymphotoxin for the sheep was prepared by the usual method, and was then injected into a rabbit in order to determine if a hæmolymphotoxin active for one animal would have the same action in an animal of a different species or if its action would be peculiar to the species for which it was prepared. Following this injection (Table X) there is an extremely marked preliminary leucopenia showing that the peritoneal irritation has been extreme. This leucopenia is followed by a well-developed leucocytosis, which is in turn followed by a decided drop in the number of leucocytes, interrupted by one slight rise; the number of leucocytes then gradually returns to normal. The amphophiles rise steadily for twenty hours, after which they as steadily decrease, rising to normal just before death. The preliminary decrease of lymphocytes is well-marked and of long duration; these cells do not begin to increase in number until the twentieth hour, after which they very closely follow the total number of leucocytes.

It would appear, then, as if proliferation in the lymphocyte-forming organs had received a check, or that amœboid movement of the fully developed cells in the lymph glands and spleen was slower or more difficult than usual.

The eosinophiles follow the usual course observed after the injection of hæmolymphotoxin, decreasing at first and then rising until the forty-fourth hour, when the so-called crisis occurs.

Changes undergone by the erythrocytes are of great interest since they give evidence of very marked hæmolytic action. There is at first a sharp rise of about one million, and then in the next five hours a decided drop of over three millions. Subsequently the number fluctuates as if the destruction at times overcame the supply, but steadily returns to normal.

RABBIT 1.—Received 10 cc. of serum from Goose 12 at 12 noon, April 23, 1904. Killed, 1 P.M., April 28. At autopsy the organs were found to be normal; there was no infection. No hæmolymph glands were found at autopsy.

Histology.—The germ centres in the lymph glands are fairly well developed, and proliferation is active. Mitotic figures are fairly numerous throughout the gland. Degenerative changes also are very abundant, many large vacuolar, vesicular, pyknotic, and fragmenting nuclei being present. Endothelial proliferation is also very marked, and many large endothelial phagocytes contain particles of pigment, probably hæmotoidin, while some contain fragments of nuclei. Eosinophiles are greatly increased in numbers.

Proliferative changes in the spleen are less evident than in the lymph glands, but there is very marked endothelial proliferation. The blood pigment here present in rather large amounts is hæmatoidin. Eosinophiles are markedly increased in numbers.

The cellular elements throughout the bone marrow are undergoing proliferation; the endothelium is also the seat of proliferation.

Comparison of the histological changes just described and those produced by the injection of hæmolymphotoxin into a sheep shows that they are almost identical.

Difference in the changes affecting the blood and perhaps the slightly more toxic effect which the serum seems to have on the rabbit may be explained by the fact that a greater amount of serum per kilo of body weight was injected into the rabbit. Moreover, the hæmolytic effect on the rabbit is very marked, a destruction of 43% of the total number of erythrocytes in circulation having occurred in five hours.

The eosinophiles of the rabbit, like those of the animal for which the hæmolymphotoxin was prepared, are increased greatly in numbers. It is probable, then, that the toxin produced by the injection of emulsions of hæmolymph glands into an intermediary animal is heterotoxic, the changes in the two species employed being the same in every respect.

SUMMARY OF EXPERIMENTS WITH HÆMOLYMPHOCYTOTOXIC SERUM.

After the injection of the serum of an animal immunized against hæmolymph glands there is a series of changes which may be classified in several groups.

I. *Changes Due to Hæmolytic Properties.*—The hæmolytic properties of the so-called hæmolymphotoxin are very marked and their effects are observable not only in the blood counts, but also in the tissues. Its hæmolytic power is in fact stronger than that of the serum obtained when the same number of corpuscles are injected into the intermediary animal as are contained in the emulsion of hæmolymph glands employed. This fact is demonstrated by the following table in which parallel experiments are compared:

(*a*) Normal serum caused an increase of 1,000,000 erythrocytes.

(*b*) 1-Hæmolytic serum caused the destruction of 3,000,000 erythrocytes, or 28% of the entire number in circulation.

2-Hæmolymphotoxic serum caused the destruction of 4,392,000 erythrocytes, or 47% of erythrocytes in circulation.

(*c*) 1-Hæmolytic serum caused the destruction of 1,060,000, or 19% of erythrocytes in circulation.

2-Hæmolymphotoxic serum destroyed 1,560,000, or 32% of erythrocytes in circulation.

There is, therefore, a difference in one case of 19% and in the other of 13% between parallel experiments. Taking into consideration the fact that in one case the goose serum may have had a stronger inherent tendency to destroy erythrocytes than in the other and the possible percentage of error, it is still impossible to explain the difference between the two experiments.

Moreover, when the serum was heated to a temperature sufficient to destroy all hæmolytic action in a serum produced by the injection of erythrocytes alone, there was still considerable destruction of erythrocytes after injection into the sheep. Furthermore, on injection of the toxin into an animal of a different species the hæmolytic effect was very marked—more marked than would usually follow the injection of hæmolytic serum produced with erythrocytes alone.

Taking all these facts into consideration, it seems probable that the hæmolymphotoxin has some hæmolytic action not due to the reaction of the goose to erythrocytes alone.

II. *Changes Due to Lymphotoxic Properties.*—Certain of the changes produced by the injection of hæmolymphotoxin are referable to the immunization of the goose against the lymphoid tissue in the hæmolymph glands.

In one experiment there is apparently an inhibition of proliferation in the germ centres, while in the other two proliferation is marked. This occurrence of proliferation agrees with results generally obtained after injection of lymphotoxin. While proliferation is not necessarily due to the direct action of the toxin, in most instances at least no inhibition of proliferation is caused by hæmolymphotoxin.

The changes affecting the leucocytes of the blood and to a certain degree the proliferative changes in the blood-forming organs are due to the peritoneal irritation following the injection of the toxin; to a certain extent they are referable to the direct effect of the toxin.

The degenerative changes which occur are, however, due to the lymphotoxic action, and those following the injection of hæmolymphotoxin are in all respects like those following the use of lymphotoxin.

III. *Changes Due to Endotheliotoxic or Phagocytoxic Properties.*—In order to demonstrate the presence of an endotheliotoxin or phagocytotoxin a comparison may be made of the number of phagocytes present in the spleen and lymph glands after the injection of hæmolymphotoxin, lymphotoxin, and hæmolysin.

Such a toxin is doubtless present in the hæmolymphotoxic serum employed for it is readily seen, even when a low-power objective is used, that the number of phagocytes present after the injection of hæmolymphotoxin is much greater than after the injection of hæmolysin. That their presence, moreover, is not secondary to the destruction of erythrocytes is shown by the fact that the number of phagocytes found after the injection of hæmolymphotoxin is greater absolutely than the number present after the injection of hæmolysin, while with hæmolymphotoxin the phagocytes are increased out of proportion to the number of red blood cells destroyed. There is further proof that this accumulation of phagocytes is not secondary to destruction of erythrocytes; Warthin (9) has described similar changes in hæmolymph glands after removal of the spleen.

It is probable, therefore, that an endotheliotoxin or phagocytotoxin is present in the serum.

IV. *Changes Due to Hæmolymphotoxic Properties.*—Is it necessary to assume the existence of a specific hæmolymphotoxin in order to explain the results obtained?

In the first place, there is produced a certain amount of hæmolysis which is not due alone to the erythrocytes injected into the goose.

One theory regarding the action of hæmolymph glands is that they are hæmolytic. Such hæmolysis may be explained in two ways: (1) it is due to a ferment, secreted by phagocytes; (2) the phagocytes take up and remove corpuscles bodily from the circulation.

If the first hypothesis is correct, it is evident that in immunizing the goose with emulsions of hæmolymph glands this destructive action upon erythrocytes must be overcome as well as the native resistance of the erythrocytes themselves; so that, in the end, the goose serum would become more destructive to erythrocytes than if the erythrocytes contained in the emulsions of hæmolymph glands had been alone injected.

The changes which occur in the sheep injected with hæmolymphotoxic serum are referable to substances produced during the reaction of the goose to the tissues injected into it, namely,

lymphoid tissue, endothelium, etc., and to the peritoneal irritation in the sheep due to the injection of the serum; hence it is unnecessary to assume the existence of a specific hæmolymphotoxin.

V. *Heterotoxic Properties.*—The serum obtained after the injection of emulsions of hæmolymph glands into an intermediary animal is heterotoxic, for when it is injected into an animal of a different species every detail of the process is duplicated and consequently it is not specific for the primary species alone.

CONCLUSIONS REGARDING HÆMOLYMPHOTOXIN.

(1) The toxin produced by the injection of emulsions of hæmolymph nodes from one species into an intermediary animal is complex in composition, having lymphotoxic, endotheliotoxic or phagocytotoxic and hæmolytic properties.

(2) These toxins when so obtained are heterotoxic, affecting other species besides that one which was used for the immunization of the intermediary animal.

(3) There is no specific hæmolymphotoxin.

(4) The chief function of the hæmolymph glands is hæmolysis; they remove partially broken-down erythrocytes from the circulation.

(5) Hæmolysis is probably brought about by a ferment produced in the hæmolymph node.

(6) This ferment is aided by phagocytes which remove the debris from the glands.

(7) The ferment is probably produced by the endothelium.

(8) In this connection it is desirable to emphasize the theory that hæmolymph glands have a part in the production of eosinophiles, the granules arising from products of disintegration of red cells. Such material is taken up by mononuclear leucocytes the nuclei of which become polymorphous. In almost every experiment there has been some destruction of erythrocytes and there has been an increase of eosinophiles in the blood and lymphoid structures in proportion to this destruction. Furthermore, when, in making special stains for eosinophiles, the cells were overstained with eosin and part of the eosin was afterward

removed, the phagocytes containing particles of erythrocytes tended to hold the stain longer than either ordinary phagocytes or normal erythrocytes.

After the preceding conclusions concerning hæmolymphotoxin have been reached, it becomes necessary to study carefully by the same methods other so-called cytotoxins in order to ascertain to what extent the changes produced by them vary from those already described and what degree of specificity each possesses.

EXPERIMENTS WITH ENDOTHELIOTOXIN.

So far as can be ascertained, no attempt has heretofore been made to produce a pure endotheliotoxin. In order to produce such a toxin the aortas of sheep were removed and the endothelium, under as nearly sterile conditions as possible, was scraped off and made into an emulsion in sterile physiological salt solution. Great care was necessary to avoid the removal of connective tissue and the entrance of blood into the emulsion.

The toxin produced by the injection of this emulsion into a goose gave, on being injected into a sheep, a rather typical blood picture (Table XI). There is at first a rather sharp leucopenia, followed by a leucocytosis, reaching its summit at the end of about twenty hours. The number of leucocytes then gradually decreases and again rises, producing a leucocytosis which, of greater height than the first, keeps its elevation for about twenty-five hours, the number then returning to normal. After the so-called "anticipatory" leucocytosis the changes in the total number of leucocytes are very closely followed by those of the lymphocytes, the amphophiles after the first rise markedly decreasing and then very gradually rising to a level somewhat above normal, to decrease again sharply.

Changes in the number of erythrocytes are noteworthy; from the first, with the exception of a slight rise at the end of the sixty-sixth hour, it gradually yet steadily decreases, falling from 5,160,000 before injection to 2,760,000 at time of death—a destruction of 2,400,000 corpuscles or over 46% of entire number of erythrocytes in circulation.

SHEEP 15.—Received 40 cc. of serum from Goose 15 at 1.30 P.M., April 9, 19. One nucleated red blood corpuscle was seen in counting five hundred cells on April 10, at 9.30 P.M. The animal was killed April 15, 10.30 A.M. At autopsy the entire body is anæmic, though fairly well nourished. A large amount of intraperitoneal fluid is present. The hæmolymph glands are numerous and hyperplastic. The liver and kidneys are normal. Spleen is slightly smaller than normal. The heart and lungs are normal. The bone marrow is fairly red. There is no infection.

Histology.—The germ centres in the hæmolymph glands are more numerous and larger than in any experiment heretofore examined. Proliferation in the germ centres is exceedingly active. Many large phagocytes filled with blood pigment or with entire red blood corpuscles are to be seen. Endothelial proliferation is very marked. Evidence of degeneration is rarely found. Eosinophiles are somewhat increased in numbers.

In the lymph glands, as well as in the hæmolymph glands, the germ centres are large, numerous, and show signs of very active proliferation. Proliferation of endothelium is also very marked, and large numbers of endothelial phagocytes containing blood pigment, probably hæmatoidin, are to be seen. On the other hand, degenerative changes are here very numerous, many vesicular, some vacuolar, and a few pyknotic nuclei being found. Eosinophiles are normal.

The spleen is slightly smaller than normal. The germ centres in the spleen are large and are the seat of active proliferation. Endothelial cells have undergone proliferation and many endothelial phagocytes containing blood pigment, probably in this organ also hæmatoidin, are to be seen. Degenerative changes are marked.

Proliferative changes in the bone marrow are widespread, every type of cell being involved in the process, which affects particularly the endothelium, so that large endothelial cells may be seen in every stage of multiplication. The myelocytes and erythroblasts are also greatly increased in number. Large numbers of mature cells such as amphophiles are present.

The liver shows a slight fatty degeneration and the kidney a slight amount of cloudy swelling.

The results obtained in the preceding experiment are important because they demonstrate the occurrence of marked changes not only in the blood but in the blood-forming organs. The marked and steady decrease in the number of erythrocytes after the injection of the toxin indicates that it is possible for the endothelium alone, through its phagocytic tendencies, to cause a profound and perhaps lasting anæmia. It cannot, however, be maintained that this destruction of erythrocytes is due to phagocytic action alone, for the histological changes will not justify such a statement. It is not improbable that a large part of it is due to the secretion of some ferment, such as that present in

the hæmolymph glands; it destroys the erythrocytes and causes their removal from the circulation.

EXPERIMENTS WITH HEPATOTOXIN.

In 1900, Delezenne (11) obtained a *serum antihepatique* by injecting emulsions of the liver of dogs into rabbits and ducks. A strongly toxic serum causing death in doses of 4 cc. per kilo of body weight was obtained. At autopsy, the liver was yellow, friable, and shrivelled, the animal having apparently died with a condition resembling that produced by phosphorus. By microscopical examination the cells were found to be markedly degenerated. In certain areas the lesion was more advanced and the protoplasm of the cells had in great part disappeared. The blood-vessels were dilated and capillaries filled with blood separated the rows of degenerated hepatic cells. There was an infiltration of leucocytes throughout the lobules. When the process was less acute, the animal lived from five to fifteen days, and marked fatty degeneration of the liver alone was noted.

Pearce (12) produced hepatotoxins with emulsions made from liver tissue washed entirely free of blood and with unwashed liver. After injection of hepatotoxins prepared with unwashed liver he found no changes in the hepatic cells which could be ascribed to their specific action. With sera from washed livers he found increased granulation of the liver cells, with slight, fatty transformation. Necrotic areas were found in only one experiment in which a large dose of strongly hæmagglutinative serum was employed; he believes that these necrotic areas are due to thrombi formed by agglutinated red blood cells.

In the experiment to be described (Table XII) there are no characteristic changes in the blood. There is a sharp preliminary leucocytosis, followed by a step-like decrease in the number of leucocytes; subsequently there are two minor rises. The first leucocytosis is altogether due to an increase in amphophiles; the subsequent leucocytoses are due to an increase of both amphophiles and lymphocytes.

The eosinophiles undergo a preliminary decrease, followed by a gradual rise and fall.

The erythrocytes exhibit rapid preliminary increase, followed by a rather sharp and well-marked fall, after which there is a return to normal, again followed by a very gradual yet distinct decrease.

SHEEP 16.—Received 35 cc. of hepatotoxic serum from Goose 16 at 1 P.M., April 5, 1904. Killed, 11 A.M., April 11. At autopsy the hæmolymph glands are found to be few in number. The spleen is slightly smaller than normal. There is an elevated white nodule, 5 x 5 x 5 mm. on the liver. The liver is light in color and apparently contains fat. The spleen is dark in color. The other organs are normal. There is no infection.

Histology.—The germ centres in the hæmolymph glands are large, and are the seat of active though not excessive proliferation. There is little evidence of degeneration, although some pyknotic and vesicular nuclei are to be seen. There is some proliferation of endothelium. Eosinophiles are decreased in number.

The lymph glands show no changes beyond a slight increase of proliferation in the germ centres and endothelium, and the presence of a considerable amount of blood pigment, probably hæmatoidin, contained for the most part within endothelial phagocytes.

The spleen is slightly smaller than normal and somewhat congested. There is increased proliferation both in the germ centres and in the endothelium. Some blood pigment is present, but it is entirely hæmatoidin, for no reaction for iron could be obtained.

The bone marrow is almost wholly normal, only a slight increase of myelocytes and erythroblasts being apparent.

The elevated white area on the liver noted at autopsy proved to be an encysted animal parasite. The liver is the seat of fairly advanced fatty degeneration. The kidney shows only a very slight cloudy swelling.

Changes in the blood, as before stated, are limited to the rather marked hæmolysis. Although an attempt was made to wash the blood from the liver before injecting it into the goose it was impossible to remove the blood entirely, so that hæmolysis is probably for the most part due to this factor. The histological changes are not characteristic; changes in the blood-forming organs are insignificant and the fatty change in the liver, while confirming the results obtained by Delezenne, is not so marked as that produced by some other cytotoxins. Nearly every cytotoxin causes at least a slight fatty degeneration of the liver. We may say, then, that the hepatotoxins have no specific action.

EXPERIMENTS WITH SPLENOTOXIN.

Flexner (3) found after the injection of spleniotoxin that the chief changes were in the lymph glands and spleen. In the lymph glands the germ centres were large and the cells had undergone degeneration, but few mitotic figures were present. In the spleen there was wide distension of the blood sinuses, slight general hyperplasia of splenic cells, and marked evidences of degeneration.

Changes in the blood (Table XIII) obtained in the following experiment are peculiar. There is at first a rather rapid rise and fall of the number of leucocytes, soon followed by a slighter rise and fall; there is then a period of about forty-eight hours during which the leucocytes vary but little; a marked increase in number occurs and is followed by a gradual decrease. The first increase is entirely due to an amphophile leucocytosis, there being at the same time the usual lymphopenia. The second increase is referable to the lymphocytes, while the third is again due to amphophiles.

The eosinophiles decrease at first and then gradually rise until the so-called crisis occurs at the end of thirty-six hours.

Changes affecting the erythrocytes are characteristic; hæmolysis causes the destruction of only 15% of the erythrocytes present in the circulating blood.

SHEEP 18.—Received 30 cc. of splenotoxic serum from Goose 18 at 1 P.M., April 14, 1904. Killed, April 21, 1.30 P.M.

Autopsy.—Hæmolymph glands are small but numerous. The spleen is smaller than normal. The kidneys are normal. The liver is light in color. The bone marrow is anæmic. Moderate amount of peritoneal fluid is present. The heart and lungs are normal. There is no infection.

Histology.—The hæmolymph glands are somewhat congested. Germ centres are few in number, and there is little proliferation of cells except in the endothelium. Degenerative changes are very numerous, and many vesicular and pyknotic nuclei are to be seen. Eosinophiles are decreased in numbers.

The germ centres in the lymph glands are sparsely scattered, but those which are found are large; they show many degenerative changes, the nuclei of the cells being swollen and vacuolar.

The spleen is small. There is a slight tendency to general hyperplasia of the lymphadenoid tissue, but more conspicuous is the marked degeneration, characterized by the presence of pyknotic and fragmented nuclei. In the

hæmolymph glands, lymph glands, and spleen numerous endothelial phagocytes are found containing blood pigment, probably hæmatoidin, and nuclear debris.

The bone marrow contains much fat, and few changes from the normal are to be observed, except perhaps proliferation of endothelium.

The liver shows advanced fatty degeneration, the liver cells being occupied by large globules of fat. The distribution of fat is fairly uniform throughout the lobules.

The kidneys show slight cloudy swelling.

The changes observed in this experiment resemble very closely those described by Flexner. The toxin apparently has a specific effect upon the spleen, causing extensive degenerative changes; these changes, however, do not differ so much from those produced by other toxins that we are justified in assuming that the difference is one of kind and not of degree. The fatty degeneration of the liver is particularly noteworthy since it is greater in extent than that found after the injection of hepatotoxin, and therefore makes still more doubtful the specificity of that toxin.

EXPERIMENTS WITH NEPHROTOXIN.

In a recent article by Pearce (12) the subject of nephrotoxins is so carefully reviewed that it will be necessary here merely to describe their action upon the blood and blood-forming organs.

Changes in the number of leucocytes (Table XIV) are characterized by a preliminary leucopenia which is of long duration. This leucopenia is followed by a well-marked rise and fall and later by two lesser rises. The behavior of the leucocytes seems to depend upon the lymphocytes for, saving a slight rise in the number of amphophiles during the first leucocytosis, the changes of the total number of leucocytes and of lymphocytes are very similar.

The erythrocytes show at first a slight increase in number, and then a gradual decrease, showing that the serum is somewhat hæmolytic in spite of the fact that a large part of the red blood corpuscles have been washed out of the emulsion before it has been injected into the goose.

SHEEP 17.—Received 40 cc. of nephrotoxic serum from Goose 17 at 2 P.M., April 1, 1904. Killed, April 7, 11 A.M.

Autopsy.—The hæmolymph glands are dark in color. The liver bleeds freely on section. There is well-marked congestion of the medullary portion of the kidney. All other organs are normal; there is no infection.

Histology.—The hæmolymph glands are fairly normal, having well-developed germ centres, which are the seat of active proliferation, and are in all respects normal. Endothelial phagocytes are somewhat more abundant than normally. They contain particles of blood pigment, probably hæmatoidin, and entire erythrocytes. Eosinophiles are normal in number. There is an increased proliferation of endothelium.

The germ centres in the lymph glands are large and actively proliferating. In the mesenteric glands in particular there is a considerable amount of blood pigment, probably hæmatoidin, contained in large phagocytes. There is considerable proliferation of endothelium shown by the presence of large, free, mononuclear endothelial cells, some of which are the phagocytes above described. The eosinophiles are slightly increased in numbers.

The spleen is little affected by the injection of nephrotoxin, and is normal save for a slightly increased proliferation of the endothelium. There is present a small quantity of free blood pigment, hæmosiderin.

The bone marrow is little altered. All the cellular elements, particularly the eosinophile cells, are undergoing proliferation, which is slightly more active than usual.

The congestion of the medullary part of the kidney, noted macroscopically, is in places so marked that the lumina of the tubules are encroached upon. There are a few hyaline casts. The cells of the tubules furthermore show some cloudy swelling and desquamation, which is most marked in the cortex, where some of the tubules are entirely filled with debris.

The changes produced by nephrotoxin in the blood and blood-forming organs are insignificant; those changes which occur, namely, proliferation in the germ centres and proliferation of the endothelium, are secondary, the one to the peritoneal irritation, and the other to destruction of erythrocytes.

The changes in the kidneys, however, are well marked and characterize the action of the serum.

EXPERIMENTS WITH LYMPHOTOXIN.

Peritoneal irritation following the injection of lymphotoxin prepared by the method already described is doubtless severe since there is a marked preliminary leucopenia (Table XV). This leucopenia is soon followed by a rather pronounced increase in the number of leucocytes, the bone marrow apparently responding to the demand upon it. The available supply having been exhausted, the leucocytes decrease and then rise again more

slowly yet to a greater height; this increase of the total number is due to an increase both of amphophiles and lymphocytes. The slow response of the lymphocytes to a demand upon them is evident since the preliminary lymphopenia is marked and of considerable duration, the increase of lymphocytes being gradual until the final leucocytosis which is almost entirely due to lymphocytosis.

The erythrocytes increase at first and then gradually return to normal. The eosinophiles and large mononuclears show no noteworthy change.

SHEEP 11.—Received 30 cc. of lymphotoxic serum from Goose 11, March 25, 1904, at 2.30 P.M. The animal was killed March 31, at 10 A.M.

Autopsy.—There is a small amount of peritoneal fluid. The hæmolymph glands are small and few in numbers. The left kidney is slightly smaller than normal. The spleen is somewhat small. Lymph glands are numerous and rather hyperplastic. There is a considerable amount of pericardial fluid. Little fat is present throughout the body. The liver is normal. There is no infection.

Histology.—The hæmolymph glands are considerably congested and contain only a small amount of lymphoid tissue. The few germ centres which are present are, however, large and show active proliferative changes. There is also considerable proliferation of endothelium. The eosinophiles are few in number, being considerably less abundant than usual. There is some pyknosis.

The germ centres in the lymph glands are numerous and large, showing many mitotic figures. There is also evident proliferation of endothelium, and large numbers of endothelial phagocytes containing blood pigment, probably hæmatoidin, are present. Evidences of degeneration are limited to the presence of pyknotic, vesicular, and a few fragmented nuclei; there are no necrotic areas similar to those found by Bunting (4) after the injection of heated hæmolymphotoxic serum. The eosinophiles are decreased in numbers.

The spleen is somewhat atrophic. The germ centres are large, numerous, and the seat of active proliferation. A considerable amount of both extracellular and intracellular pigments is present, and is in great part, if not entirely, hæmosiderin. There is little evidence of degeneration. Eosinophiles are decreased in number.

The cellular elements of the bone marrow are markedly depleted. Those which are present are, however, undergoing marked proliferation. The myelocytes exhibit most active proliferation, the erythroblasts stand next in order, and the eosinophiles show the fewest mitotic figures. Fully-developed cells are few in numbers, but a few amphophiles are present.

The changes just described differ markedly from those obtained by Bunting, and resemble more closely those observed

by Flexner. They do not have a specific character, nor do they vary greatly from the changes observed after the injection of other cytotoxins.

SUMMARY OF CYTOTOXIC EXPERIMENTS.

Are the cytotoxins specific — that is, do they individually produce characteristic changes or do the changes which they produce differ in degree alone?

(1) Changes in the blood observed in the preceding experiments are in no instance characteristic and may be explained by the action on the blood and blood-forming organs, of the peritoneal irritation produced by the injection of the toxin. These changes do not vary sufficiently from those obtained by the injection of salt solution and of normal serum to justify the assumption that they show, in any case, anything specific, particularly when we take into consideration the different conditions under which the animals may have been at time of injection.

(2) Histologically, certain differences are evident, but again all of these are of degree and not of kind. For instance, fatty degeneration of the liver was found after injection of hepatotoxin, but in almost every case examined there was slight fatty degeneration, while in the case of splenotoxin the fatty change was much greater than after hepatotoxin.

In every experiment almost the same changes were observed; they may be classified as follows:

Hæmolymph Glands.—Proliferation in the germ centres and endothelium; large numbers of endothelial phagocytes containing blood pigment, probably hæmatoidin; rather marked degenerative changes.

Spleen.—Proliferation in the germ centres and endothelium; some phagocytes containing blood pigment and usually more or less free iron-containing pigment, probably hæmosiderin.

Bone Marrow.—Proliferation of all elements.

Kidney.—Slight cloudy swelling.

Liver.—Slight fatty degeneration.

Throughout the entire series of injections these changes are

to be observed and they vary merely in degree. All the cytotoxic sera are therefore hæmolytic, endotheliolytic, lymphotoxic, myelotoxic, etc.

When we take into consideration the severity of the changes which occur, the various sera may be arranged in the following order:

(*a*) Hæmolymphotoxin,
(*b*) Splenotoxin,
(*c*) Lymphotoxin,
(*d*) Endotheliotoxin,
(*e*) Nephrotoxin,
(*f*) Hepatotoxin.

It is readily seen that the above order takes into consideration the amount of hæmolysis produced by each serum. In the case of the spleen and the hæmolymph glands it is practically impossible to remove this factor, and in making the above classification the other factors have been given greater importance than this one. We have, then, in every case, a serum which causes a certain series of changes which always vary as to degree but not as to kind.

(3) The nephrotoxin perhaps comes nearest to being specific, since macroscopically and microscopically marked congestion of the medullary portion of the kidney and pronounced cloudy swelling in the cortex were observed. Similar changes of the same severity were found with no other serum, and it appears that the renal toxin had a specific, perhaps merely irritating, action on the kidney itself.

(4) It seems, furthermore, that the hæmolymph glands play some important part in the production of eosinophile cells, for in no case was there such a marked increase of these cells as after the injection of hæmolymphotoxin; indeed, after most of the toxins used, the number of eosinophile cells was found decreased.

CONCLUSIONS REGARDING CYTOTOXINS.

(1) The cytotoxins are not specific.

(2) The changes occurring after the injection of different cytotoxins are similar and do not vary in kind.

(3) The changes do, however, vary in degree in the following order, the one having the most marked effect being placed first, etc.:

(*a*) Hæmolymphotoxin,
(*b*) Splenotoxin,
(*c*) Lymphotoxin,
(*d*) Endotheliotoxin,
(*e*) Nephrotoxin,
(*f*) Hepatotoxin.

(4) Nephrotoxin causes the most specific change.

(5) The hæmolymph glands play some important part in the production of eosinophiles. The hæmolytic action of hæmolymphotoxic serum is greater than that of other cytotoxic sera.

(6) The histological changes which are observed in every case are secondary to the hæmolytic factor present in all cytotoxic sera.

In conclusion I wish to express my thanks to Dr. Warthin, in whose laboratory and under whose directions these experiments were conducted, for his aid and encouragement in the work.

DESCRIPTION OF PLATES.

Plate IV.

Fig. 1.—Area of necrosis in lymph gland of Sheep 4 after injection of heated hæmolymphocytotoxin. Stained with hæmatoxylin and eosin.

Fig. 2.—Increase of eosinophile cells in hæmolymph gland of Sheep 3 after injection of hæmolymphocytotoxin. Mononuclear, polymorphonuclear, and polynuclear eosinophiles are found in the lymphadenoid tissue of the hæmolymph gland. Stained with hæmatoxylin and eosin.

Plate V.

Fig. 3.—Hæmatoidin in lymph gland of Sheep 3 after injection of hæmolymphocytotoxic serum. Stained with hæmatoxylin and eosin.

Fig. 4.—Iron-containing pigment found in spleen after injection of hæmolymphocytotoxin, demonstrated by the Prussian-blue reaction. Counter-stained with borax carmine.

BIBLIOGRAPHY.

1. Warthin.—*Trans. Chicago Path. Soc.*, 1902, v, 151.
2. Lewis.—*Jour. of Anat. and Phys.*, 1902, xxxviii, Appendix, p. vi.
3. Flexner.—*Univ. of Penn. Med. Bull.*, 1902, xv, 287.
4. Bunting.—*Univ. of Penn. Med. Bull.*, 1903, xvi, 200.
5. Muir.—*Jour. of Path. and Bact.*, 1901, vii, 161.
6. Besredka.—*Annales de l'Institut Pasteur*, 1900, xiv, 390.
7. Ricketts.—*Trans. Chicago Path. Soc.*, 1902, v, 178.
8. Brinckerhoff and Tyzzer.—*Jour. of Med. Research*, 1902, viii, 449.
9. Warthin.—*Contrib. to Med. Research*, Dedicated to Dr. V. C. Vaughan, 1903, p. 216.
10. Dayton.—*Am. Jour. of Med. Sciences*, cxxvii, 448.
11. Delezenne.—*Compt. Rend. de l'Acad. de Sciences*, 1900, cxxxi, 427.
12. Pearce.—*Univ. of Penn. Med. Bull.*, 1903, xvi, 217.
13. Pearce.—*Jour. of Med. Research*, 1904, xii, 1.

TABLE I.

Time.	Feb. 14, 10 A.M. Before injection.		Feb. 14, 2.30 P.M. 3½ hours after injection.		Feb. 14, 10.30 P.M.		Feb. 15, 8.30 A.M.		Feb. 15, 8.30 P.M.		Feb. 16, 8.30 A.M.		Feb. 16, 8.30 P.M.		Feb. 17, 8.30 A.M.	
	Per cent.	*Total.*	*Per cent.*	*Total*	*Per cent.*	*Total.*	*Per cent.*	*Total.*	*Per cent.*	*Total.*	*Per cent.*	*Total.*	*Per cent.*	*Total.*	*Per cent.*	*Total.*
Lymphocytes	32.2	4,079	26.4	3,627	31.5	5,065	36.7	4,646	37.5	5,108	33.6	5,080	34.	4,821	30.8	4,035
Large Mononuclears	7.2	912	6.5	893	5.8	933	7.3	924	8.6	1,171	8.1	1,225	6.4	908	8.	1,048
Amphophiles	52.6	6,662	63.	8,656	58.7	9,439	52.1	6,596	44.1	6,006	52.1	7,878	51.8	7,345	52.	6,812
Eosinophiles	8.	1,013	3.9	536	4.	643	3.7	469	9.8	1,335	6.	907	7.8	1,106	8.4	1,100
Mast Cells	0	0	.2	28	0	0	.2	25	0	0	.2	30	0	0	.8	105
Total Leucocytes	100	12,666	100	13,740	100	16,080	100	12,660	100	13,620	100	15,120	100	14,180	100	13,100
Erythrocytes	7,500,000		7,000,000		7,500,000		7,000,000		6,800,000		6,800,000		7,200,000		7,200,000	

Differential count after injection of cold sterile physiological salt solution.

TABLE II.

Time.	Dec. 1, 1 P.M. Before injection	Dec. 1, 7 P.M. 4 hrs. after injection.	Dec. 1, 12 P.M.	Dec. 2, 9 A.M.	Dec. 2, 4 P.M.	Dec. 3, 8 A.M.	Dec. 3, 8 P.M.	Dec. 4, 9 A.M.	Dec. 5, 9 A.M.	Dec. 6, 9 A.M.	Dec. 7, 9 A.M.
Lymphocytes	4,735	6,504	4,791	5,137	4,833	7,023	7,058	7,156	5,256	5,558	4,725
Large Mononuclears	410	537	285	475	1,076	1,045	919	1,097	546	854	824
Amphophiles	4,035	3,606	6,689	13,911	13,520	7,023	4,178	5,128	2,845	2,694	2,974
Eosinophiles	140	86	95	178	117	1,209	1,338	1,364	754	894	919
Mast Cells	0	0	0	59	20	33	27	75	19	50	28
Total Leucocytes.	9,320	10,733	11,860	19,760	19,566	16,333	13,520	14,820	9,420	10,050	9,470
Erythrocytes	12,000,000	12,800,000	13,340,000	14,240,000	13,640,000	13,620,000	13,640,000	13,380,000	14,100,000	13,200,000	14,520,000

Differential count after injection of normal serum of the goose (Sheep 2).

TABLE VII.

Time.	Apr. 23, 11 A.M. Before injection.	Apr. 23, 5 P.M. 3 hrs. after injection.	Apr. 23, 10 P.M.	Apr. 24, 10 A.M.	Apr. 24, 10 P.M.	Apr. 25, 10 A.M.	Apr. 25, 10 P.M.	Apr. 26, 10 A.M.	Apr. 27, 10 A.M.	Apr. 28 10 A.M.
Lymphocytes	3,077	2,424	3,444	3,394	3,120	3,178	2,775	2,385	2,863	2,506
Large Mononuclears	690	603	806	666	1,040	609	623	525	618	688
Amphophiles	3,105	2,955	3,571	4,226	3,760	2,820	3,962	4,515	3,158	3,910
Eosinophiles	0	12	32	17	32	0	15	15	27	14
Mast Cells	28	36	47	17	48	13	45	60	54	42
Total Leucocytes	6,900	6,030	7,900	8,320	8,000	6,620	7,420	7,500	6,720	7,160
Erythrocytes	5,100,000	4,820,000	5,070,000	5,200,000	5,340,000	4,280,000	4,350,000	4,600,000	5,080,000	5,180,000

Differential count after injection of hæmolytic serum (Sheep 14).

TABLE VIII.

Time.	Nov. 18, 11 A.M. Before injection.	Nov. 18, 7 P.M. 4 hrs. after injection.	Nov. 18, 12 P.M.	Nov. 19, 9 A.M.	Nov. 19, 8 P.M.	Nov. 20, 9 A.M.	Nov. 20, 4 P.M.	Nov. 21, 9 A.M.	Nov. 22, 9 A.M.	Nov. 23, 9 A.M.
Lymphocytes	3,953	3,200	3,827	5,148	3,486	3,977	6,595	5,693	3,320	5,606
Large Mononuclears	533	508	373	616	458	512	567	513	318	283
Amphophiles	2,326	4,458	4,531	5,461	3,856	4,959	7,296	2,781	3,320	6,258
Eosinophiles	374	167	169	372	233	372	432	308	275	148
Mast Cells	14	0	0	23	0	0	30	38	0	25
Total Leucocytes	7,200	8,333	8,900	11,620	8,033	9,820	14,920	9,333	7,233	12,320
Erythrocytes	9,600,000	8,540,000	9,560,000	9,220,000	9,640,000	9,460,000	9,440,000	9,630,000	9,300,000	8,860,000

Differential count after injection of heated hæmolymphocytotoxic serum (Sheep 4).

TABLE IX.

Time.	May 16, 10 A.M. Before injection.	May 16, 4 P.M. 3 hrs. after injection.	May 16, 9 P.M.	May 17, 9 A.M.	May 17, 9 P.M.	May 18, 9 A.M.	May 18, 9 P.M.	May 19, 9 A.M.	May 20, 9 A.M.	May 21, 9 A.M.
Lymphocytes	6,484	5,312	6,739	7,680	8,660	7,962	7,530	6,733	6,752	5,984
Large Mononuclears	1,138	864	1,152	1,220	1,205	1,076	1,511	1,210	1,060	1,091
Amphophiles	1,687	1,728	1,498	1,262	893	1,162	1,906	1,814	1,153	1,408
Eosinophiles	38	48	153	316	380	517	604	262	260	246
Mast Cells	133	48	58	42	22	43	69	61	75	71
Total Leucocytes	9,480	8,000	9,600	10,520	11,160	10,760	11,620	10,080	9,300	8,800
Erythrocytes	12,290,000	12,950,000	10,800,000	11,460,000	11,890,000	12,180,000	11,940,000	12,400,000	12,020,000	12,000,000

Differential count after injection of heated hæmolymphotoxic serum (Sheep 19).

TABLE X.

Time.	Apr. 23, 9 A.M. Before injection.	Apr. 23, 3 P.M. 3 hrs. after injection.	Apr. 23, 8 P.M.	Apr. 24, 8 A.M.	Apr. 24, 8 P.M.	Apr. 25, 8 A.M.	Apr. 25, 8 P.M.	Apr. 26, 8 A.M.	Apr. 27, 8 A.M.	Apr. 28, 8 A.M.
Lymphocytes	4,485	2,033	1,075	1,776	5,433	3,126	3,080	2,096	3,028	4,037
Large Mononuclears	607	455	202	365	664	563	940	629	985	765
Amphophiles	1,573	2,275	4,233	6,026	3,863	3,126	3,403	2,347	1,775	1,949
Eosinophiles	138	48	56	83	60	141	46	84	110	42
Mast Cells	97	29	34	58	40	84	231	84	182	167
Total Leucocytes	6,900	4,840	5,600	8,300	10,060	7,040	7,700	5,240	6,080	6,960
Erythrocytes	6,670,000	7,740,000	4,420,000	6,400,000	5,200,000	6,060,000	5,890,000	6,050,000	5,760,000	6,260,000

Differential count after injection into a rabbit of hæmolymphotoxic serum prepared for sheep.

TABLE XI.

Time.	Apr. 9, 11 A.M. Before injection.	Apr. 9, 4.30 P.M. 3 hrs. after injection.	Apr. 9, 9.30 P.M.	Apr. 10, 9.30 A.M.	Apr. 10, 9.30 P.M.	Apr. 11, 9.30 A.M.	Apr. 11, 9.30 P.M.	Apr. 12, 9.30 A.M.	Apr. 13, 9.30 A.M.	Apr. 14, 9.30 A.M.	Apr. 15, 9.30 A.M.
Lymphocytes.....	4,563	2,997	3,018	4,798	5,219	4,144	4,128	4,805	5,643	4,569	3,965
Large Mononuclears.	607	349	338	683	618	602	564	574	756	558	520
Amphophiles.....	2,365	3,101	3,960	2,832	2,004	2,184	2,064	2,201	2,785	3,488	1,976
Eosinophiles.....	45	13	15	17	16	0	110	62	18	35	13
Mast Cells.....	0	0	29	0	63	70	14	108	18	70	26
Total Leucocytes..	7,580	6,460	7,360	8,330	7,920	7,000	6,880	7,750	9,220	8,720	6,500
Erythrocytes.....	5,160,000	5,020,000	4,320,000	4,000,000	3,620,000	3,360,000	3,280,000	3,800,000	3,620,000	3,040,000	2,760,000

Differential count after injection of endotheliotoxic serum (Sheep 15).

TABLE XII.

Time.	Apr. 5, 10 A.M. Before injection.	Apr. 5, 4.30 P.M. 3½ hrs. after injection.	Apr. 5, 10.30 P.M.	Apr. 6, 9 A.M.	Apr. 6, 9 P.M.	Apr. 7, 9 A.M.	Apr. 7, 9 P.M.	Apr. 8, 9 A.M.	Apr. 9, 9 A.M.	Apr. 10, 9 A.M.	Apr. 11, 9 A.M.
Lymphocytes.....	3,147	1,399	1,616	2,435	3,304	3,193	3,454	2,761	3,780	3,786	4,336
Large Mononuclears.	543	318	231	423	552	377	490	490	378	689	671
Amphophiles.....	1,706	4,116	9,652	7,122	5,644	3,511	2,679	1,931	2,772	2,204	3,059
Eosinophiles.....	111	12	23	60	294	145	163	138	70	67	98
Mast Cells.....	33	35	23	20	39	14	14	0	0	14	16
Total Leucocytes..	5,540	5,880	11,545	10,060	9,833	7,240	6,800	5,320	7,000	6,760	8,180
Erythrocytes.....	6,420,000	7,440,000	7,020,000	6,800,000	5,500,000	5,800,000	6,060,000	6,020,000	5,620,000	4,920,000	4,000,000

Differential count after injection of hepatotoxic serum (Sheep 16).

TABLE XIII.

Time.	Apr. 14, 11 A.M. Before injection.	Apr. 14, 4 P.M. 3 hrs. after injection.	Apr. 14, 9 P.M.	Apr. 15, 9 A.M.	April 15, 9 P.M.	Apr. 16, 9 A.M.	Apr. 16, 9 P.M.	Apr. 17, 9 A.M.	Apr. 18, 9 A.M.	Apr. 19, 9 A.M.	Apr. 20, 9 A.M.	Apr. 21, 9 A.M.
Lymphocytes	2,866	1,843	1,995	2,762	3,771	3,480	3,496	3,414	2,451	3,443	3,288	4,730
Large Mononuclears	461	186	367	510	505	696	654	553	548	653	499	836
Amphophiles	1,883	5,431	3,746	2,343	2,311	1,812	1,736	1,883	3,197	3,294	7,484	5,390
Eosinophiles	10	0	12	34	53	12	48	24	12	15	23	22
Mast Cells	10	0	0	11	0	0	12	12	12	15	46	22
Total Leucocytes	5,230	7,460	6,120	5,660	6,640	6,000	5,946	5,886	6,220	7,420	11,340	11,000
Erythrocytes	7,180,000	6,920,000	7,300,000	7,500,000	7,820,000	7,320,000	6,580,000	6,780,000	6,800,000	7,200,000	6,800,000	7,000,000

Differential count after injection of splenotoxic serum (Sheep 18).

TABLE XIV.

Time.	Apr. 1, 1 P.M. Before injection.	Apr. 1, 5 P.M. 3 hrs. after injection.	Apr. 1, 12 P.M.	Apr. 2, 9.30 A.M.	Apr. 2, 6.30 P.M.	Apr. 3, 9.30 A.M.	Apr. 3, 7.30 P.M.	Apr. 4, 8.30 A.M.	Apr. 5, 8.30 A.M.	Apr. 6, 8.30 A.M.	Apr. 7, 8.30 A.M.
Lymphocytes	4,180	3,270	2,800	5,581	5,183	5,532	3,487	4,923	3,739	5,460	5,163
Large Mononuclears	562	413	552	1,243	860	734	716	926	566	575	740
Amphophiles	3,303	3,881	2,751	3,673	2,083	1,714	2,885	2,313	3,025	2,873	2,269
Eosinophiles	198	46	37	381	99	131	36	49	15	36	32
Mast Cells	17	30	0	22	41	49	36	49	15	36	16
Total Leucocytes	8,260	7,640	6,140	10,900	8,266	8,160	7,160	8,260	7,360	8,980	8,220
Erythrocytes	9,720,000	10,100,000	9,200,000	9,640,000	8,820,000	9,000,000	9,000,000	9,200,000	9,320,000	9,000,000	8,800,000

Differential count after injection of nephrotoxic serum (Sheep 17).

TABLE XV.

Time.	Mar. 25, 2 P.M. Before injection.	Mar. 25, 5.30 P.M. 3 hrs. after injection.	Mar. 25, 10.30 P.M.	Mar. 26, 8.30 A.M.	Mar. 26, 8.30 P.M.	Mar. 27, 8.30 A.M.	Mar. 27, 8.30 P.M.	Mar. 28, 8.30 A.M.	Mar. 29, 8.30 A.M.	Mar. 30, 8.30 A.M.	Mar. 31, 8.30 A.M.
Lymphocytes.....	2,857	1,491	1,470	2,300	2,699	2,491	2,242	3,067	2,767	4,235	2,965
Large Mononuclears.	379	136	124	289	305	202	235	270	262	494	349
Amphophiles.....	2,970	2,616	4,542	5,427	3,681	2,586	4,387	3,720	4,421	5,468	3,894
Eosinophiles.....	101	17	44	24	221	21	56	43	30	62	43
Mast Cells.....	13	0	0	0	14	0	0	0	0	21	15
Total Leucocytes..	6,320	4,260	6,180	8,040	6,920	5,300	6,920	7,100	7,480	10,280	7,266
Erythrocytes.....	4,426,000	5,080,000	5,240,000	5,660,000	4,960,000	5,740,000	4,960,000	4,960,000	5,120,000	4,980,000	4,580,000

Differential count after injection of lymphotoxic serum (Sheep 11).

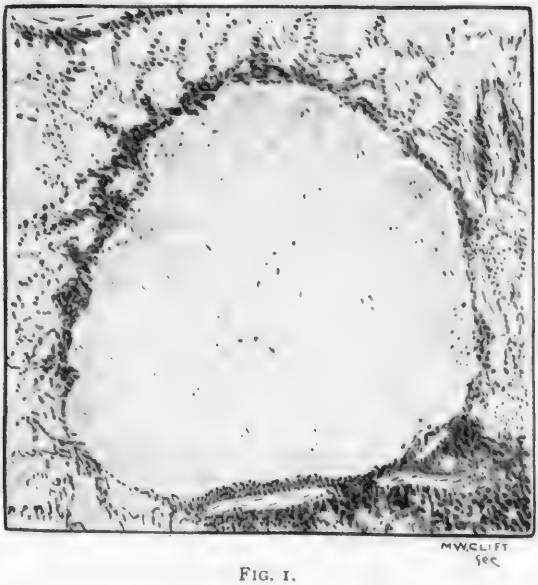

FIG. 1.

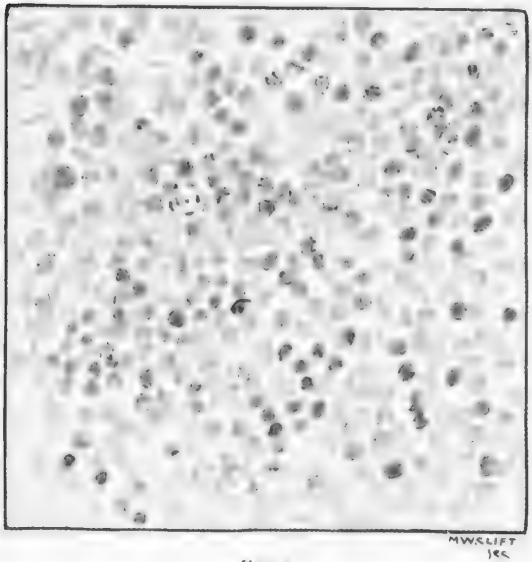

FIG. 2.

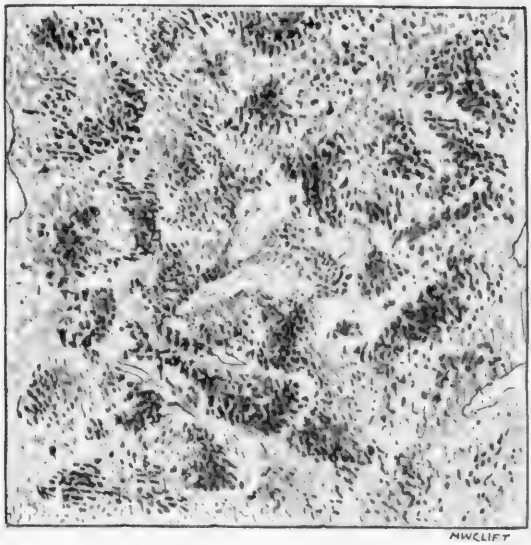

FIG. 3.

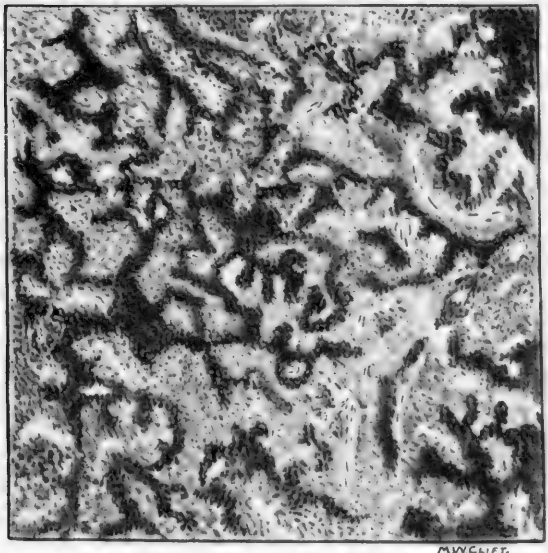

FIG. 4.

THE ARTIFICIAL ANASTOMOSIS BETWEEN THE PORTAL VEIN AND THE VENA CAVA INFERIOR—ECK'S FISTULA.[1]

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Plates VI–VIII.

It is the purpose of the following paper to discuss briefly the methods which have been used in the operation for directing the blood of the portal vein into the vena cava, and to describe a new instrument for cutting the opening between the veins. The operation is named after N. V. Eck (1), who conceived the idea of establishing an artificial anastomosis between the portal vein and the vena cava, in order to relieve the congestion of the portal system in cirrhosis of the liver. Never attempted upon the human being, the operation performed on dogs has yielded a great part of the experimental facts concerning the normal physiology of the liver, and still offers an invaluable method for approaching many problems of metabolism. I shall leave aside for the present the question of the changes in the animal economy which follow the diversion of the portal blood current, simply appending a list of the literature bearing upon the subject.

The necessity of a stringent asepsis during a surgical procedure requiring between one and two hours for its completion is self-evident. The unusual difficulties encountered are peculiar to this operation and are purely of a mechanical nature: one is referable to the inaccessibility of the site of the operation—that portion of the portal vein extending about five to eight centimeters below the opening of the vena pancreatico-duodenalis, and the somewhat longer section of the vena cava between the

[1] Read before the American Association of Pathologists and Bacteriologists, April, 1904.

hepatic and the renal veins; another is due to the extreme thinness and delicacy of the walls of the portal vein; but the chief difficulty is that of cutting an opening in the coapted walls of the two vessels within an oblong space bounded, except at one or two points, by a row of firmly tied sutures.

All who have successfully performed the operation have followed essentially the same technique up to the cutting of the fistula opening, except in the method devised by Queirolo (6) and later modified by von Karltreu (18, 22). Queirolo's operation has already been sufficiently criticised (7); von Karltreu's modification may correct the fault of Queirolo's method, but the distortion of the channels through which the blood must pass, and the traction upon the vena pancreatico-duodenalis are manifest disadvantages, and the operative results obtained by von Karltreu indicate that they are more than mere disadvantages. The question of the fact of the distance between the vena pancreatico-duodenalis and the first branchings of the portal vein into the liver[2] is one which every one can settle for himself. In my own experience it depends entirely upon the dog. The method described by Tansini (23) has not received the impress of experimental results; I do not believe it possible, because of the inaccessibility of the site of the operation.

The details of my operation are as follows. A dog—the size is immaterial—is anæsthetized by a mixture of one part chloroform to four parts ether; the abdomen is shaved and scrubbed and washed with 4 % carbolic acid; the dog and the operating table are then entirely covered with sterilized towels, the towel covering the dog's abdomen being made to adhere slightly by dampening with sterile salt solution. Towels, instruments, suture material, etc., are sterilized in the autoclave. An incision of sufficient length is then made in the median line, and the field of operation exposed by an assistant who holds the intestines toward the splenic side of the peritoneal cavity. The first step is the placing of the ligature around the portal vein below its first branchings into the liver and above the opening of the vena pancreatico-duodenalis, the tying of which, immediately

[2] See von Karltreu (22), p. 63.

after the fistula opening has been made, will constitute the final step in the operation. The portal vein is then freed of the subperitoneal fat, if it be not already free for a sufficient extent, by blunt dissection.

An oval of the desired size, about three centimeters long by six to ten millimeters wide, is then mentally planned and a row of sutures is laid along the side of this oval nearer the spleen (Fig. 1). The sutures should be about three or four millimeters apart. The delicacy of the wall of the portal vein makes it necessary to pass the needles each time into the lumen of the vessel, a procedure which makes it essential that the needles be of extremely small size, and that the silk be of such nature as to fill completely the hole.

The needles which we have used are fashioned from the smallest size (No. 15) of round needle found on the market for use in bead work. They are made with a long eye, which greatly facilitates threading. The temper is drawn, the needles are cut to about two thirds their original length, sharpened to a very fine, round point, bent to a half curve, and then re-tempered. The needle-holder used is a Halstead mosquito hæmostat. The needles carry a fine, loose silk thread obtained by untwisting the two strands of the finest black embroidery silk; the resultant thread is composed of many filaments of silk, but is not twisted; used double, it fills completely the holes left by the needles; the needles are, however, so fine that the wall of the portal may be punctured by them with impunity. The wall of the vena cava is of such thickness that no difficulty is experienced even with larger needles. It is found most convenient to place all the sutures along one side of the oval before tying any of them, the two ends of each suture being caught with a hæmostat to prevent an entanglement of the various threads; after all are placed, the sutures are tied, the vessels being readily brought together by the act of tying the sutures.

The next step is to place in position in each vein the silk threads by means of which the cautery wire is later to be drawn into place (Fig. 1). The cautery wire consists of a fine platinum wire, made pliable by annealing, and provided with an eye at

each end. Long silk threads of the same size as those used for the sutures are passed through each eye, and both ends of each thread then passed through the eye of a long, slightly curved needle. These needles must, because of their length, be larger than those used for placing the sutures, and the silk thread which they carry must be correspondingly thicker than that used for the sutures, in order to fill completely the holes made by the needles in the vessels. This is attained by passing both ends of the threads in the cautery wire through the eyes of the needles, and drawing them through for a sufficient distance to allow the needles, with the four-ply thread, to be drawn into and out of the vessels far enough to be out of the way of the subsequent steps (Figs. 2 and 3). One needle is inserted into the lumen of the portal vein at a point two or three millimeters below the end of the oval nearer the liver, and brought out of the portal at a point the same distance from the lower end of the oval; the other needle is introduced in the same manner into the vena cava (Fig. 1).

The oval is now completed by placing another series of sutures (Fig. 2), which are not tied until the next step in the operation—the drawing of the cautery wire into position by traction on the silk threads (Fig. 3)—has been performed. Since the cautery wire is of smaller diameter than the silk threads, some slight bleeding may occur at this time, but it soon stops, either by coagulation or after the next step in the operation—the tying of the sutures just laid (Fig. 4). The bleeding may be prevented entirely by tying the second series of sutures, except the one at either end of the oval, before drawing the cautery wire into place. We have usually, however, disregarded this hæmorrhage, which is rarely more than a mere oozing of blood, for the sake of having the assurance that the cautery wire is in its proper position. After tying the sutures of the second row, a suture is laid around the cautery wires (Figs. 3, 4, and 5) to close completely the lower end of the oval after cutting the opening. The threads are cut from the cautery wires, and the two ends of the wire threaded into the cautery (Fig. 5). The current is regulated by a simple water rheostat, which is set before the

operation so that a platinum wire of the same size as the cautery wire is heated white-hot; it is usually found necessary to increase the strength of the current, which is done by an assistant, until the operator feels the yielding of the tissue due to the cutting by the cautery. As soon as the cautery wire frees itself from the tissues, the current is turned off, the cautery is removed, and the suture at the lower end of the oval is tied. The ligature laid around the portal as the first step in the operation is now tied; the operation is complete.

The abdominal wound is closed by a row of interrupted sutures through the *linea alba* and the peritoneum, and by interrupted sutures or a continuous suture of the skin. The wound is dressed with iodoform or bismuth-formic-iodide and covered with gauze dressing pads. The dog is then tightly bandaged, the bandage being covered with a muslin shirt—a piece of muslin of sufficient length to extend from the neck to the hind legs, provided with two holes through which the fore legs are passed, the ends being cut into narrow strips, which are tied along the middle of the dog's back. This bandage is left in place for a week or ten days. Bitches are chosen for the operation, because the male dog cannot be prevented from soiling the bandage with urine, and thus infecting the wound.

I mention these minor details because Pawlow writes as follows[3]: "A misfortune which has often occurred has pursued us during the whole course of our operations: six to seven days after a successful operation the edges of the wound separated. If this occurred during the night we usually found our animals dead in the morning. Sometimes we could help ourselves by immediately sewing the abdomen together again. It is to be hoped that later workers succeed in avoiding this truly annoying mishap by the use of another method of suturing."

I have not had such a misfortune in my short experience with the operation; indeed, the wounds have healed in nearly every case without even a stitch abscess. I have thought this result due to the use of a tight bandage rather than to the method of suturing.

[3] 3 (2), p. 167.

The construction of the cautery is shown in the diagram (Fig. 6). Two small German silver tubes (Fig. 6, a–b, a–b), insulated from each other, through which the cautery wire will easily pass, are soldered to insulated wires (Fig. 6, c, c). The insulation of the German silver tubes and the conducting wires is best accomplished by making the cautery of vulcanized fibre—the only material in our hands which has successfully withstood the heat transmitted from the platinum wire. The free ends of the cautery wire naturally remain cold; one end is held by the hand which holds the cautery in position, while traction with the other hand on the other end of the cautery wire draws the white-hot wire through the walls of the veins.

I believe that the use of the electro-cautery offers several distinct advantages over the best instrument hitherto used—Pawlow's modification of Eck's scissors. The points of these small scissors are soldered to long silver wires, the ends of which are soldered to curved needles. In regard to these scissors Pawlow himself says: "A certain number of animals succumbed because the walls of the veins were torn with the needles or with the wire ends of the scissors. Sometimes the operation did not succeed if the ends freed themselves from the blades of the scissors after the upper row [4] of sutures was already placed. In general the faulty cohesion of the soldered joint between the silver thread and the scissors is the weak side of our instrument, and it is only to be wished that one could find a better method of soldering, as well for the result of the operation as for the operator's peace of mind." [5]

In addition to avoiding this difficulty in the construction of Pawlow's scissors—which has been obviated, at least to a certain extent, by Italian workers, who substituted a copper wire for the silver wire—I believe the method above described offers at least two very distinct advantages. By the use of the soft silk threads in placing the cutting instrument in position, the danger of cutting the veins by a wire of any kind, during the considerable manipulation necessary for the placing of the second row of sutures, is entirely avoided. In the second place, the fact

[4] The second row.

[5] Loc. cit., p. 167.

that the cutting instrument—the cautery wire—can be placed in actual position for cutting before the second row of sutures is tied permits the assurance that everything is in its proper position, and also makes it possible to tie all the sutures at the upper end of the oval before cutting the fistula opening. In Pawlow's method openings of sufficient size to permit the passage of the scissors must be left at both ends of the oval—openings through which a very considerable hæmorrhage may occur before the sutures placed ready for tying can be knotted. ("In the majority of cases the hæmorrhage is rather considerable, maximum sixty ccm." [6])

The use of the cautery makes it possible to close the upper end of the oval entirely, while the nose of the cautery occludes the lower end. The maximum hæmorrhage in our experience was about ten cubic centimeters, while ordinarily no hæmorrhage at all occurred. A further distinct advantage is that the cautery wire is so pliable that it can be introduced no matter how small the space is between the liver and the oval described by the sutures; while in Pawlow's method a space equivalent to the length of the scissors must be left between the lowest point of the liver and the highest point of the oval, since, whether the scissors be introduced from above or from below, they cannot be introduced or removed on a curve. The free portions of the veins are so limited, especially in small dogs, that any increase of available space is very welcome.

The question of the influence of the heat of the cautery upon the coagulation of the blood and the consequent formation of a thrombus in the fistula opening is one that I can answer only by referring to the results of my operations. The first six animals upon which the operation was performed, after the method described above was perfected, recovered perfectly and were utilized for further studies. Some of the results obtained have already been reported by Dr. P. B. Hawk, of the University of Pennsylvania (24).

The seventh dog, operated upon by Dr. H. J. Nichols, of Washington, D. C., at that time a fourth-year student in the

[6] Loc. cit., p. 166.

University of Pennsylvania, recovered satisfactorily from the operation, but died three or four days later from an infection with an anaërobic, capsulated bacillus. Our operating-room had become infected with this micro-organism, and we lost six or seven other dogs from which the pancreas had been removed, from the same infection. An animal upon which I recently operated died during the following night for some reason which the autopsy did not satisfactorily reveal; the lungs showed extensive infiltration, but it was not determined whether the infiltration was to be ascribed to pneumonia or to embolism. The organs of the portal system showed no evidences of congestion. It is fair to state that the animal was in very poor condition before the operation was performed.

A ninth dog, recently operated upon, has recovered satisfactorily from the operation, making in a total of nine dogs, seven successful operations, one death from infection, and one death from what may have been the effects of the narcosis.

The work described above was performed at the Laboratory of Hygiene of the University of Pennsylvania, to the Director of which, Professor Abbott, I am indebted for every aid he could possibly extend me. I am further greatly indebted to my friend, Dr. C. R. Turner, Professor of Prosthetic Dentistry at the University of Pennsylvania, to whose patient teaching I owe the necessary knowledge of the working of metals. I would also express my thanks to Drs. Stewart, Hawk, Fager, Lewis, and Nichols, who acted as anæsthetists and assistants during the operations.

DESCRIPTION OF PLATES.

PLATE VI.

Fig. 1.—First row of sutures tied. Silk thread in place with which cautery wire is to be drawn into position.

Fig. 2.—Second row of sutures in position.

PLATE VII.

Fig. 3.—Cautery wire drawn into place.

Fig. 4.—Second row of sutures tied, except the safety suture around the cautery wire.

PLATE VIII

Fig. 5.—Cautery wire threaded into cautery.

Fig. 6.—Construction of cautery. a–b, a–b, German silver tubes. c, c, wires insulated from each other.

BIBLIOGRAPHY.

1. Eck, N. V.—*Militär-medicinisches Journal*, 1877, cxxx, Jahrgang 55. Also in *Travaux de la Soc. des Naturalistes de St. Petersburg* (*Bulletins de la Section Zoologique*), 1879, x.

2. Stolnikow.—"Die Stelle vv. hepaticarum im Leber- und gesammten Kreislaufe." *Pflüger's Archiv für die gesammte Physiologie*, 1882, xxviii, 255.

3. (1) Hahn, Massen, Nencki et Pawlow.—"La fistule d'Eck de la veine cave inferieure et de la veine porte et ses consequences pour l'organisme." 1. Partie physiologique, par MM. Massen et Pawlow. 2. Partie chimique, par MM. Hahn et Nencki. *Archives des sciences biologiques de St. Petersbourg*, 1892, i, 401. Also in (2) "Die Eck'sche Fistel zwischen der unteren Hohlvene und der Pfortader und ihre Folgen für den Organismus." *Archiv f. exp. Path. u. Pharm.*, 1893, xxxii, 161.

4. Pawlow, J. P.—"Sur une modification dans l'operation de la fistule d'Eck entre la veine porte et la veine cave inferieure." *Archives des sciences biologiques de St. Petersbourg*, 1893, ii, 581.

5. Kotliar.—"Contribution à l'étude du rôle du foie comme organe defensif contre les substances toxiques." *Archives des sciences biologiques de St. Petersbourg*, 1893, ii, 587.

6. Queirolo.—"Ueber die Function der Leber als Schutz gegen Intoxication vom Darm aus." Moleschott's *Untersuchungen zur Naturlehre des Menschen und der Thiere*, 1895, xv, 228.

7. Nencki.—Footnote in regard to the method of Queirolo, in Maly's *Jahresbericht über die Fortschritte der Thierchemie*, 1895, xxv, 310.

8. Nencki, Pawlow und Zaleski.—"Ueber das Ammoniakgehalt des Blutes und der Organe und die Harnstoffbildung bei den Säugethieren." *Archiv f. exp. Path. u. Pharm.*, 1898, xxxvii, 26. Also in "Sur la richesse du sang et des organes en ammoniaque et sur la formation de l'urée chez les mammifères." *Archives des sciences biologiques de St. Petersbourg*, 1896, iv, 191.

9. Hédon et Delezenne.—"Effets des injections intraveineuses de peptone après exstirpation du foie combinée à la fistule d'Eck." *Compt. rend. de la Soc. de Biol.*, 1895, xlviii, 633.

10. Magnamini.—"La modificazioni del ricambio azotato dopo l'innesto della vena porta colla vena cava inferiore." *Il Policlinico*, 1896, iii, 153.

11. Magnamini.—"Les modifications de l'échange azoté après qu'on a mis la veine porte en communication avec la veine cave inferieure." *Arch. ital. de biologie*, 1896, xxvi, 66.

12. Schupfer.—"Sugli effeti che induce nell' organismo l'innesto della vena porta nella vena cava inferiore per rispetto all' autointossicazioni di origine intestinale." *Il Policlinico*, 1896, iii, 357.

13. de Filippi.—"Nota sul ricambio materiale dei cani operati di fistula Eck." *Riforma med.*, 1896, xii (vol. iv), 243.

14. Nencki und Pawlow.—"Zur Frage über den Ort der Harnstoffbildung bei den Säugethieren." *Archiv f. exp. Path. u. Pharm.*, 1897, xxxviii, 215.

15. Popelski, L.—"Ueber das Schicksal des Zuckers bei Hunden mit Eck'scher Fistel." *Bolnitschnaja gaseta Botkina*, 1897, No. 46. Also see *St. Petersburger med. Wochenschrift*, 1898, xxiii, Beilage 1.

16. Salaskin, S.—"Ueber das Ammoniak in physiol. und patholog. Hinsicht und die Rolle der Leber im Stoffwechsel stickstoffhaltiger Substanzen." *Zeitschrift für physiologische Chemie*, 1898, xxv, 449.

17. Bozzi.—"Alterazioni anatomopatologiche nei cani operati di fistola di Eck." Bologna, 1898.

18. v. Karltreu.—"Demonstration und Mittheilung über die Vereinigung der unteren Hohlvene mit der Pfortader (Eck'sche Fistel)." *Wiener klin. Wochenschrift*, 1899, xii, 205.

19. de Filippi.—"Recherches sur l'échange matériel des chiens opérés de fistule d'Eck. Contribution à l'étude de la physio-pathologie du foie." *Archives ital. de biologie*, 1899, xxxi, 211.

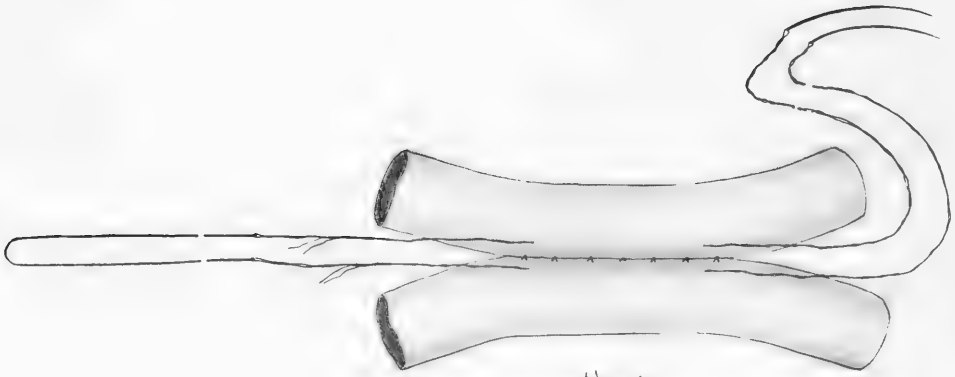
20. Salaskin und Zaleski.—"Ueber den Einfluss der Leberexstirpation auf den Stoffwechsel bei Hunden." *Zeitschrift für physiologische Chemie*, 1900, xxix, 516.

21. Bozzi.—"Richerche sperimentali dirette a studiare i rapporti possibili fra intestino, fegato e reni." Firenze, 1900.

22. v. Karltreu.—"Ueber die Vereinigung der unteren Hohlvene mit der Pfortader." *Arch. f. exp. Path. u. Pharm.*, 1901, xlv, 56.

23. Tansini.—"Ableitung des portalen Blutes durch directe Verbindung der V. portae mit der V. cavae. Neues operatives Verfahren." *Centralblatt für Chirurgie*, 1902, xxix, 937.

24. Hawk, P. B.—*American Journal of Physiology*, 1905, xiii, 14.



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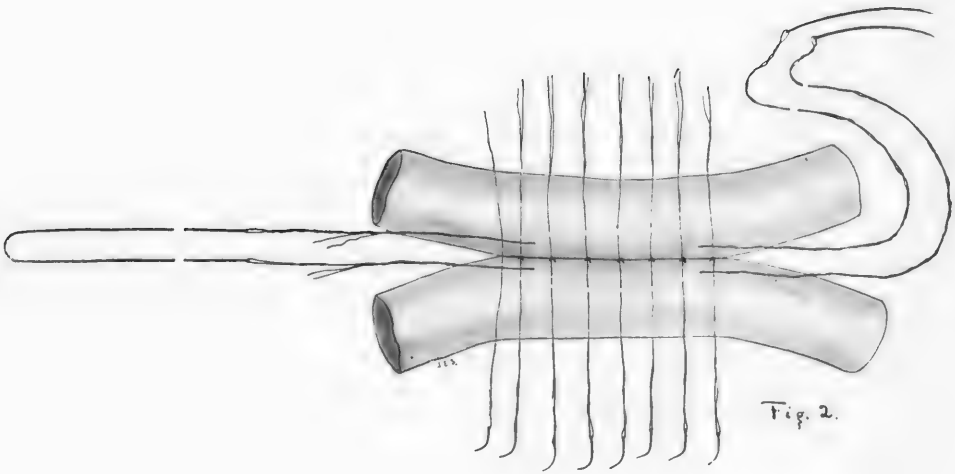

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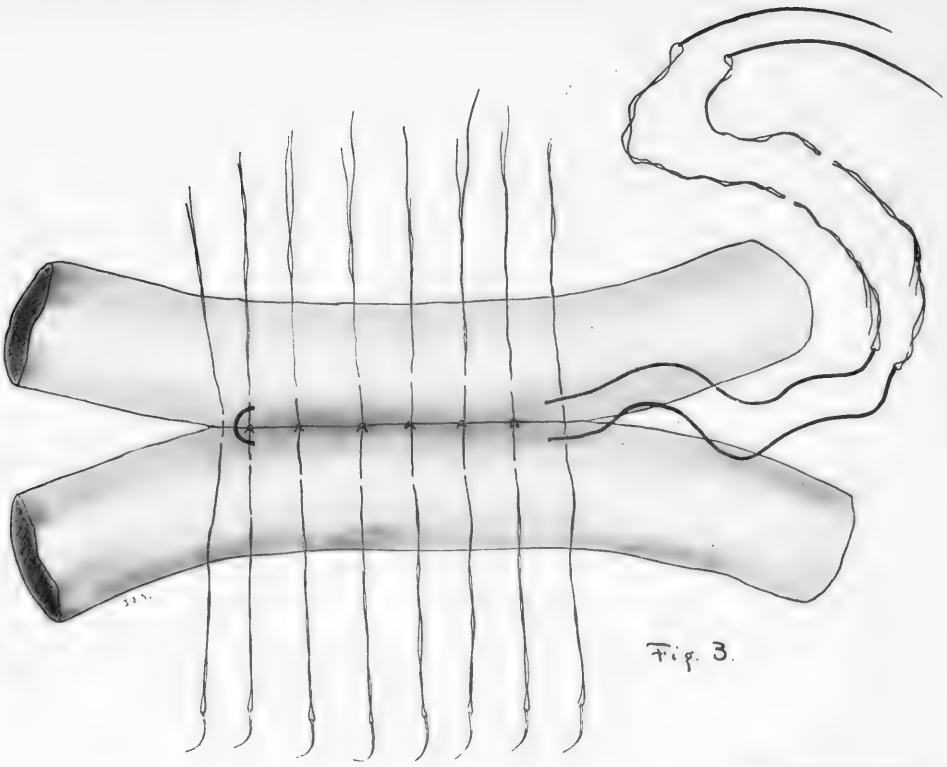

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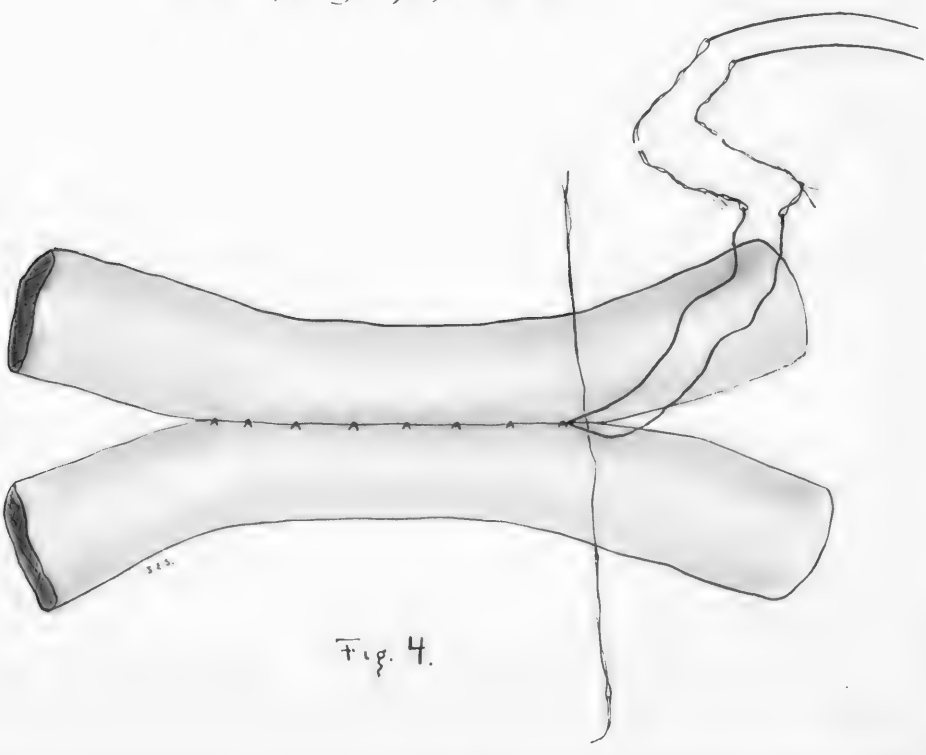

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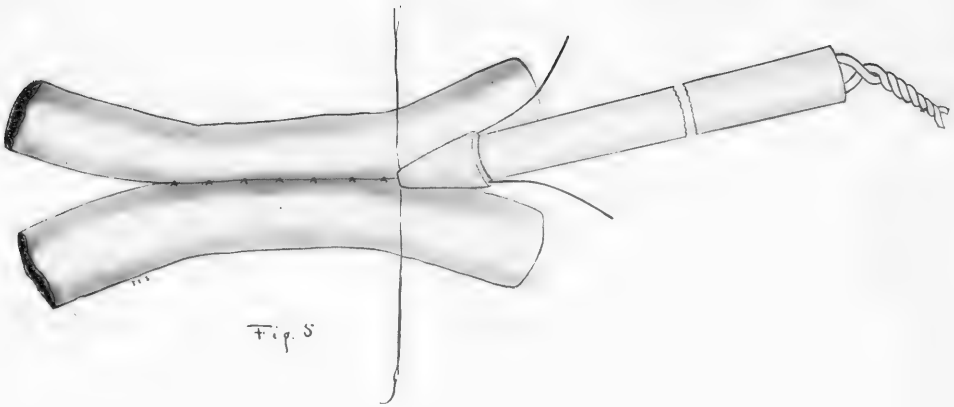


Fig. 5

Fig. 6

A SECOND COAGULATION OF THE BLOOD DUE TO A SUBSTANCE THAT IS NOT IDENTICAL WITH FIBRINOGEN AND IS COAGULABLE BY SATURATION WITH NEUTRAL OXALATE.

By EDWARD T. REICHERT, M.D.

(*From the Physiological Laboratory of the University of Pennsylvania.*)

That the oxalated bloods of certain species may undergo coagulation, even in the presence of an excess of oxalate, has been known for some years. A plausible explanation has been suggested in the probability that the oxalate does not cause the plasma to become lime-free, but leaves sufficient lime salts in solution to be slowly effective. It was during an investigation of this problem that I discovered that the oxalate may not only hinder or prevent coagulation, but actually cause it; and that the oxalate is anticoagulative to one substance and coagulative to another.

The experiments were carried out chiefly with the bloods of the *necturus* and *amia*, which bloods were oxalated in the usual proportion, or defibrinated.

If to the oxalated blood either neutral ammonium or potassium oxalate be added to saturation, in the course of several minutes, the time varying with the freshness of the blood and other conditions, a thickening begins which gradually increases until there is formed a solid, very elastic, and slightly contractile coagulum. The question at once arose as to whether or not the coagulation is due to the plasma-fibrinogen, or to another substance, or to both. To determine this point the fibrinogen was removed from the plasma by beating, and then the oxalate was added to saturation. This defibrinated blood was found to coagulate as before defibrination, thus showing that the coagulative principle that is caused to undergo coagulation by saturation

with oxalate cannot be, unless in minor part, the plasma-fibrinogen. To prove if the oxalate to saturation has a coagulative action on the plasma-fibrinogen, oxalated blood was centrifugalized, and the clear plasma then saturated with oxalate, with a negative result. Having thus determined that the plasma-fibrinogen is not the coagulative substance affected by the oxalate, it seemed clear that a second principle exists which is not only not identical with the plasma-fibrinogen, but which belongs to the corpuscles. Tests made with centrifugalized blood rendered it obvious that the chief or sole source must be in the red cells. The upper layer of corpuscles, rich in colorless cells and poor in red cells, reacted to saturation with oxalate poorly, if at all; the middle layer, poor in colorless cells and rich in red cells, reacted well; and the lowermost layer, which consisted essentially of red cells, yielded a coagulum more quickly and of firmer consistency than even the normal blood. Moreover, that the coagulative substance is contributed by the red cells is further indicated by the fact that when the cytoplasm of these cells is caused to disintegrate, as by agitation with ethyl ether, coagulation occurs more promptly, and the coagulum is firmer and more elastic than when the corpuscles are practically intact. This observation also shows that the clot formed in defibrinated blood is not merely an agglutination of the corpuscles caused by the deposition of some substance of the plasma, or from the colorless cells, but is due to a principle of the red cells.

My experiments with mammalian bloods, although as yet very limited, indicate that two clot-forming substances, one coagulable by saturation with neutral oxalate and the other not, may be common to all or nearly all bloods, and that the corpuscular principle, like the plasma-fibrinogen, varies in percentage not only in the bloods of different species, but in the bloods of the same species, and even in the bloods of different vessels of the same individual.

That the red corpuscles yield such a coagulative material seems evident also from a number of facts, as, for instance: The red corpuscles of certain mammals have been found to yield a fibrinogenous substance; the fibrinogenous material of the

bloods of certain amphibia and birds is stated to originate in the breaking down of the red cells; the quantity of fibrin formed in mammalian blood is greater when the red corpuscles are present than when absent; agents which cause a partial or complete disintegration of the red cells increase the rapidity of clotting and the quantity of fibrin formed, etc. In connection with the foregoing it is of interest to note that the results of recent research indicate that the chief source of the plasma-fibrinogen is probably the leucocytes, especially those of the intestinal tract.

The fact that the red cells undergo gradual dissolution in the shed blood, and even with considerable rapidity in certain bloods, makes it probable that the coagulative substances of both plasma and red corpuscles constitute together, in some cases at least, the normal fibrin-yielding material, the proportions of the two varying in different bloods under different conditions. This view is in accord with such facts as pertain to the differences in the composition of the fibrins obtained from the plasma and from the whole blood; the differences in the fibrins from the bloods of different species, and from the bloods of different vessels of the same species; and the occurrence of coagulation of bloods which contain an ordinary amount (1:1000), or an excess, of oxalate.

The fact that the fibrinogen of the plasma is not coagulated by saturation with neutral oxalate, and that the coagulable substance of the red corpuscles is so coagulated, can scarcely leave a reasonable doubt as to their non-identity.

COMPARATIVE STATISTICS OF ANTITOXIN HORSES. A STUDY OF THE RECORDS OF ONE HUNDRED HORSES IMMUNIZED TO DIPHTHERIA TOXIN, WITH COMPOSITE OF CURVES.

By WILLIAM R. HUBBERT, M.D., Detroit, Mich.

Although there is no method known by which we are able to select high-test horses in advance of the tedious immunizing process, statistics show, nevertheless, that some advance has been made. Up to 1896 no records are available. From 1897 to 1899 the records of a few horses show that 80 % tested below 300 units and 10 % above 500 units.

During the next period, 1900 to 1902, of 54 horses injected, 64 % tested below 300 and 25 % over 500 units.

The table gives the data of the last one hundred consecutive horses partially or completely immunized in the production of antitoxin, and includes twenty horses which either died or were sold before a test drawing of blood could be made. None of these last horses was able to attain to the maximum amount of toxin injection as compared with producing animals. Several of them were sold within two months of the date of purchase. With these omitted, a better showing could be made. The table does not include the earlier animals prior to Horse 56, many of which, like Horse 41, were high-test animals. As will be seen from the table, a horse attains its maximum antitoxic value within from two to ten months from the beginning of treatment, the average being about four months. It may continue to yield serum of marketable value for a few months longer (5.1 months is the average), when the decline begins, usually never to rise again. Some notable exceptions to this rule have been made. If the serum of a new horse does not test 300 units by the end of the fourth month after the beginning of treatment, it may be regarded as nearly certain

that the animal is not a suitable one for the work. A horse is usually at his best during the fourth month of immunization. I do not consider that the data given of the average length of time horses continue to produce antitoxic serums as stated in the table fairly represent the facts. In many of the averages given the horses are still under the treatment, and probably some of them will continue to yield potent antitoxin for some months or years to come.

Atkinson [1] has found from a study of the records of the horses of the Board of Health of New York City that there seems to be a maximal antitoxic limit of each horse beyond which the animal cannot be forced, no matter how much toxin is injected, and I am able to confirm this finding.

Horses attaining a high antitoxic strength exhibit a longer period of usefulness than do lower testing horses, but usually at the expense of a much lower unit value, as will be seen by reference to the table.

The amount of toxin necessary to maintain a horse at its normal antitoxic capacity varies from 60,000 to 90,000 fatal guinea-pig doses per injection, the horses being injected usually two to three times per week.

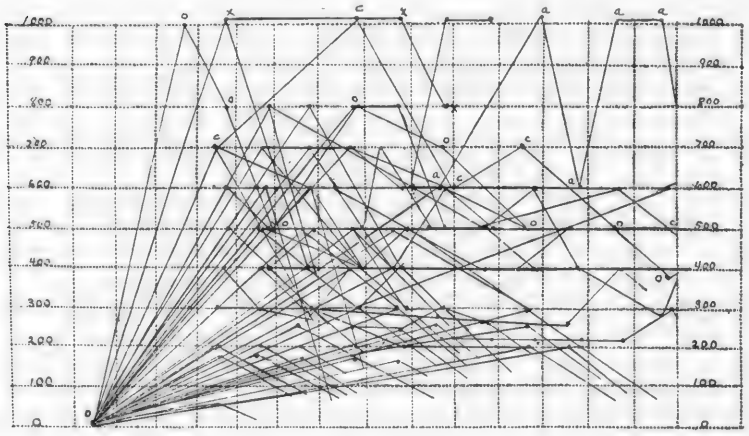
The results of the record have been attained by what are known as low-test toxins, namely, toxins ranging in lethal dose from $\frac{1}{200}$ to $\frac{1}{300}$ cc. per 250 grams of guinea-pig.

In the composite figure the antitoxic type curves of the last one hundred horses immunized to diphtheria toxin are given together with the curve of Horse 41. For the purpose of comparison all curves are represented as starting at the same time. Duplicate curves are not given. Each space from left to right represents a month of treatment and the upward curve indicates the number of antitoxic units present at any particular time.

Each dot in the curve represents a test point. The dots in the ascending limb of the curve show that at the time the tests were made the animal had reached a point at which it was able to withstand the injection of maximal amounts (300 to 700 cc.) of toxin as compared with immune horses. It will be noticed that

[1] *Journal of Medical Research*, 1903, iv, 173.

in nearly every case a decline in antitoxic strength began at this point, although the injections were continued without intermission. In a few cases the antitoxic strength increased beyond this point. The amount of toxin was rarely increased, and no attempts were made to force the animals, since it had been noticed that horses gave longer periods of service when the injections were not pushed beyond certain limits. Moreover, they



rarely died during treatment. The point at which the curves terminate indicates the time at which the horses were of no further use.

The striking feature of the composite is the uniformity with which the curves descend after having attained the maximum of antitoxic strength, and that the descent takes place despite a continuation of the immunization process. It is also noteworthy that the decline from maximum strength occurs with about the same rapidity with which the original gain in maximum activity was made. Almost without exception every rise to the maximum shows its corresponding decline, and, indeed, with almost the same inclination with which the ascension has been made. In the great majority of curves, the angle of declination equals the angle of ascension. The decline is continued until the animal has reached its normal antitoxic value, which in the case

of a high-test animal is usually high enough for therapeutic uses, and is about one half its maximum antitoxic value. A horse that takes a year to reach 700 units remains as long in service as does a horse that reaches 1000 units within three months. Both are good types. After having attained a given height, nearly all horses almost at once drop to a lower antitoxic level. It is very rarely that any of these horses ever again reach the antitoxic height from which it has dropped. A horse that takes a year to reach 200 units is of no practical value.

In the immunization of Horse 41 [2] (*oaaa*), a period of ten months elapsed before the horse attained its maximum antitoxic strength. During the first eight months the antitoxin was stored up very slowly, but in the two following months the process was more rapid. The immunization was begun in July, 1901, and has been continued up to the present time—November, 1904—a period of three and a half years. The serum of Horse 41 has touched the thousand-unit mark six different times during that period. By increasing the amount of toxin 10,000 guinea-pig fatal doses more than the average, it was always possible to increase the antitoxic content of the serum of this horse from three to four hundred units per cubic centimetre without any additional inconvenience to the animal.

The curve of Horse 113 (*occc*) is interesting. This horse rose during three months to 700 units with ease, and it was with some difficulty that during the next three months it was forced beyond this point. Probably as a result of this the antitoxin curve came rapidly down with hardly an appreciable reascension until it nearly touched the 300-unit mark, when a slight rise was again manifested.

Horse 114 (*oxxx*) is still more interesting. It was immunized within a period of sixty days from the time of purchase, and attained a maximum strength of 1000 units per cubic centimetre, when it descended as rapidly as it had ascended originally until the 400-unit mark was reached. At this point the descent was checked in its downward course and the blood has remained at that strength until the present.

[2] Not included in the table.

Horse 164 (*oxxx*) was immunized in ninety days, and barring the usual midsummer drop, still continues to yield a 1000-unit serum, although occasionally dropping to 900 units or even lower at times.

A glance at the darkened portion of the composite clearly shows how short the period of usefulness of an antitoxin horse really is. It is at best on the average but a few months, though now and then a horse may last two or three years. In this respect the statistics of the table and the composite agree identically.

The number of guinea-pig doses given in the table represent averages, and are of no great importance as compared with individual cases. One horse (89) required as little as 521,500 fatal doses to develop 600 units, while another horse (84) required the enormous amount of 4,320,400 such doses before it reached the same number of units. The average amounts of toxin injected have been slightly more than given in Atkinson's figures.[3]

Horse 114, which developed 1000 units per cubic centimetre within two months from the beginning of treatment, was able to withstand without any great difficulty from 600 to 700 cc. of toxin injected three to four times per week.

The length of time required to develop antitoxin of maximal strength has averaged slightly longer with me than with Atkinson. I think the difference arises from my not having forced the horses. This fact may also have had something to do with the greater percentage of high-test horses given in my table, although the average length of time the horses continued to produce active serums is slightly in favor of Atkinson's results.

It has been found that it takes a longer time to immunize low-test animals than high-test ones; a 300-unit horse requires on an average 6.2 months to develop its maximal antitoxic strength, while horses capable of developing 1000 units average only 3.6 months, thus, nearly one half as long a time.

By reference to the table it will be seen that the statistics of the last fifty horses are far better than those given for the entire one hundred horses. This is mainly due to the more perfect

[3] Loc. cit.

TABLE OF ONE HUNDRED CONSECUTIVE DIPHTHERIA HORSES.[4]

Horses producing diphtheria antitoxic serum. Last 100 consecutive diphtheria horses used (not all were tested).	Below 300 units per cc.	Above 300 units per cc.	Above 400 units per cc.	Above 500 units per cc.	Above 600 units per cc.	Above 700 units per cc.	Above 800 units per cc.	Above 900 units per cc.	Above 1000 units per cc.
Number of horses producing diphtheria antitoxic serum (maximum strength).....	47	11	6	14	6	9	4	—	3
Average of guinea-pig fatal doses given to produce maximum antitoxin strength.....	—	1,158,327	1,011,033	1,321,560	1,568,366	1,414,313	1,385,120	—	1,526,666
Average period of injection developing maximum strength..	4.4 months	6.2 months	5.1 months	5 months	6.1 months	6.5 months	4.2 months	—	3.6 months
Average length of time horses continued to produce antitoxin testing as per table....	—	7.1 months	5.2 months	4 months	3.9 months	3.7 months	3.7 months	—	9 months

Per cent. of horses producing serum below 300 units per cc. = 47
" " " " " " above 300 " " " " = 53
" " " " " " " 400 " " " " = 42
" " " " " " " 500 " " " " = 36
" " " " " " " 600 " " " " = 22
" " " " " " " 700 " " " " = 16
" " " " " " " 800 " " " " = 7
" " " " " " " 1000 " " " " = 3

[4] If the statistics of the last 50 consecutive diphtheria horses be taken, the results are far better than those given for 100 horses, and are as follows:

— 300 units 20 horses = 40 %
++ 300 " 30 " = 60 %
+++ 400 " 24 " = 48 %
++++ 500 " 22 " = 44 %
+++++ 600 " 12 " = 24 %
++++++ 700 " 10 " = 20 %
+++++++ 800 " 5 " = 10 %
++++++++ 1000 " 3 " = 6 %

methods used with the last horses. Since its foundation, to date, the laboratory has immunized upwards of 150 horses.

CONCLUSIONS.

1. Better results in the production of diphtheria antitoxin can be obtained with greater experience in the selection of the most suitable type of horses to be used. Young animals are usually to be preferred. Over one-half of all such horses can be made to yield 300-unit serum, while a third will yield 500-unit serum.

2. High-test horses require a shorter time to immunize and will yield a potent serum for a longer period than will low-test horses.

3. The period of usefulness of an antitoxin horse is short, and on an average endures only a few months.

4. A horse having attained a maximal antitoxic height begins to suffer a decline in antitoxin, which is usually as rapid as the ascent has been, and is unaffected by subsequent injections of diphtheria toxin.

SOME OBSERVATIONS UPON ENDOTHELIAL SEPARATION IN THE SMALLER ARTERIES AND VEINS.

By CHARLES G. FARNUM.

(*From the Pathological Laboratory of Rush Medical College.*)

Plates IX and X.

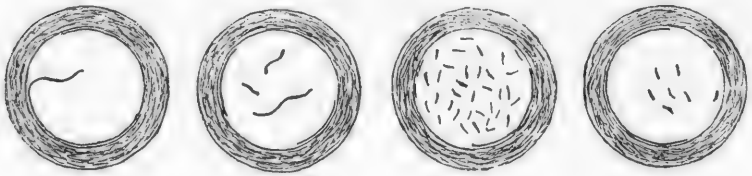
Endothelial separation is a subject which appears to have received very little attention. At the first meeting of the German Pathological Society[1] this subject was discussed by Heller. He described "peculiar findings of endothelial separation" which he had observed in the arterioles and venules, and which he believed to be a vital process because the cells lay embedded among red blood corpuscles. Schmorl stated that he had seen this condition and had formerly believed it an intra-vital one, but now he considers it a post-mortem change, because if intra-vital, emboli of endothelial cells would be found. Several others agreed with the statement of Schmorl, and Chiari remarked that in a gangrenous lung he had once found almost all the endothelium separated from the walls of the vessels.

The following observations are based upon a study of specimens and records of 164 post-mortems in the Pathological Laboratory of Rush Medical College. About half of the specimens were hardened in alcohol and formalin, the others in Zenker's fluid; most of them were embedded in celloidin, stained with eosin and hæmatoxylin, and mounted in Canada balsam.

The endothelial separation observed is not at all uniform, varying from individual cells to large plates of endothelium, and from a few cells to a number almost or quite filling the lumen of the vessel, many cells being free in the lumen, others still attached to the vessel wall. This has made it seem wise to

[1] *Centralblatt für Allgemeine Pathologie und Pathologische Anatomie*, 1899, x, 824.

attempt an arbitrary classification based upon these variations as follows:



1st. Degree. Portions of endothelium separated in entirety, but still attached to the wall of the vessel. (Fig. 1.)

2d Degree. Portions of endothelium separated in entirety and unattached to the vessel wall. (Fig. 2.)

3d Degree. Individual endothelial cells but in large numbers, almost or quite filling the lumen of the vessel. (Fig. 3.)

4th Degree. Individual endothelial cells, few in number and free in the lumen of the vessel. (Fig. 4.)

It was soon discovered that this classification could not be made along hard and fast lines, for while in most instances the degrees were distinct, cases occurred where they merged so indefinitely one into another that it was not easy to draw the line. With one or two minor exceptions, the order of frequency has been the same in all cases: separation of the 4th degree occurring most often, that of the 3d degree next, and that of the 1st degree least often.

The total number of post-mortems from which sections were examined is 164.

The total number of cases showing endothelial separation in one or more parts is 106 (65%).

Of these 106 cases, 34 show endothelial separation of 1st, 38 in the 2d, 45 in the 3d, and 76 in the 4th degree.

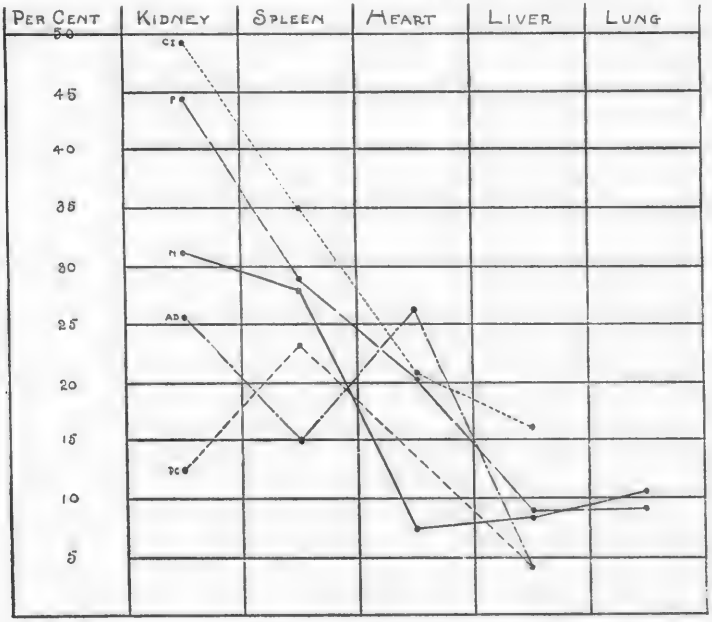
In order to bring out more clearly the frequency of endothelial separation in the different organs, certain of these organs have been studied separately. And in order to ascertain whether or not the condition bears any relation to pathological changes in these organs, they have been divided into groups according to the pathological conditions present in them, and the frequency of all separation and of each degree of separation has been

determined in each case. This form was not carried through for each pathological condition found, but only the most common have been taken and those which represent the greatest diversity of pathological changes. In some cases broad classifications have been adopted to include under one head several conditions, which are but varying degrees of one general process. For example: The term "acute degeneration" is used to include all acute degenerative processes up to an actual necrosis of the part and from whatever cause. In other cases the classification has been more narrow and specific, as brown atrophy of the heart and lobar pneumonia.

	Kidney.	Spleen.	Heart.	Liver.	Lung.
Total number examined	149	125	143	149	139
Endothelial separation	67	36	24	13	19
1st Degree	18	5	2	3	1
2d "	20	6	2	5	1
3d "	21	12	7	2	6
4th "	32	23	16	6	16
Number of normal organs	16	32	40	24	19
Endothelial separation	5	9	3	2	2
1st Degree	2	..	..	..	..
2d "	2	3	1	..	..
3d "	2	4	1	1	1
4th "	3	5	1	1	1
Number showing pathological changes	133	93	103	125	120
Endothelial separation	50	27	21	11	11
1st Degree	14	5	2	3	1
2d "	15	3	1	5	1
3d "	16	8	6	1	5
4th "	29	18	15	5	15
Number showing acute degeneration	59	20	19	44	..
Endothelial separation	25	3	5	2	..
1st Degree	5	..	..	1	..
2d "	6	1	1	..	..
3d "	11	1	1	1	..
4th "	11	1	3	..	..
Number showing chronic interstitial changes	65	37	20	25	..
Endothelial separation	32	13	6	4	..
1st Degree	9	2	1	1	..
2d "	9	3	..	1	..
3d "	4	5	3	..	..
4th "	17	9	4	2	..
Number showing passive congestion	8	21	..	46	..
Endothelial separation	1	5	..	4	..
1st Degree	..	..	..	..	..
2d "	..	..	..	..	..
3d "	1	2	..	..	..
4th "	1	4	..	4	..
Number showing segmentation or fragmentation of heart muscle	..	..	77	..	..
Endothelial separation	..	..	17	..	..
1st Degree	..	..	2	..	..
2d "	..	..	1	..	..
3d "	..	..	4	..	..
4th "	..	..	13	..	..

	Kidney.	Spleen.	Heart.	Liver.	Lung.
Number showing brown atrophy of the heart...	..	..	12	..	..
Endothelial separation.....	..	..	2	..	..
1st Degree.....	..	..	1	..	..
2d "	..	..	..	..	..
3d "	..	..	..	..	..
4th "	..	..	1	..	..
Number showing lobar pneumonia.....	..	..	..	..	30
Endothelial separation.....	..	..	..	..	5
1st Degree.....	..	..	..	..	..
2d "	..	..	..	..	..
3d "	..	..	..	..	1
4th "	..	..	..	..	5
Number showing active tuberculosis of the lungs.	..	..	..	..	28
Endothelial separation.....	..	..	..	..	6
1st Degree.....	..	..	..	..	1
2d "	..	..	..	..	1
3d "	..	..	..	..	2
4th "	..	..	..	..	4

The following chart shows the frequency of endothelial separation in the various conditions of the organs:



CI ..... Represents chronic interstitial inflammations.
P —— —— —— —— " pathological conditions in general.
N —————— " normal organs.
AD —— - —— - —— - —— " acute degenerations.
PC ---------- " passive congestion.

From the above summary it will be seen that endothelial separation occurs in a large per cent of all post-mortems (65 % in this series), in all organs and in all conditions of the organs, and with but slightly increased frequency in organs showing pathological changes as compared with the normal. In acute degenerations the heart is the first in the order of frequency, showing endothelial separation in 26.3 % of cases, while the spleen shows but 15 %, though normal spleens show this condition in 28.1 % of cases and normal hearts in but 7.5 %. It will also be seen that the frequency of this condition bears a more or less constant relation to the general condition of the part (though no pronounced relation seems to exist between it and specific conditions) and to the organ concerned. For example: In acute degenerations of the organs, the heart excepted, endothelial separation occurs in each one of them less frequently than in normal organs and very much less frequently than in those organs showing chronic inflammatory changes. And, with the exception of the heart, in acute degenerative conditions the kidney and spleen occupy the first places in the order of frequency in each condition, whether normal or pathological. Whether or not this apparent greater frequency of endothelial separation in the kidney and spleen is due to the greater number of arterioles and venules which show in sections of these organs is difficult to say and probably an accurate determination could not be made. It is possible that this may explain a part of this increase. But while this increase in the number of vessels may be a factor in explaining the greater frequency of the condition in some organs and some conditions it does not explain why endothelial separation should occur more frequently in normal organs than it does in those showing acute degeneration. Nor does any explanation which has suggested itself satisfactorily explain this finding, unless it be that the vitality of the endothelial cells has been impaired by the virulence of the toxins which caused the acute degenerative condition, while in chronic inflammatory conditions they have been stimulated to proliferation by the weakness or dilution of the toxins, as suggested by Mallory,[2] thus producing a condition which would

[2] *The Journal of Experimental Medicine*, 1900, v, 1.

favor desquamation during the death agony or immediately after death.

Since the greater number of arterioles and venules offers a possible explanation of the apparent increased frequency of endothelial separation in certain organs and in certain pathological conditions of those organs; since there is no specific condition, with the possible exception of acute myocarditis, in which endothelial separation stands out with marked prominence, though it does seem to bear some relation to general pathological conditions; and since it occurs in so large a per cent of all cases, it seems necessary to look for general explanations of the causes of separation of the endothelium.

In this, as in much work based upon microscopical findings, the question of artefacts must be considered. That a certain amount of endothelial separation might in some cases be produced during the preparation of the sections and especially during the hardening process is not at all unlikely. But this objection cannot be maintained against that very large proportion of cases in which the desquamated endothelium lies embedded among red blood corpuscles; for here it could have occurred only while the blood was fluid. Moreover, if the condition is the result of artefact it should probably be more frequent in this series; for the technique, while by no means faultless, was practically the same in all cases, with the exception of the different hardening fluids used, and there is no essential difference in the frequency of the condition in the first half of the cases when alcohol and formalin were used and the last half when the specimens were hardened in Zenker's fluid. And the hardening process certainly seems the most likely time for endothelial detachment.

In considering Heller's belief that the process is a vital one and his reason for believing so, several things suggest themselves. In the first place, if the process occurs during life we should expect to find in post-mortems showing endothelial separation, numerous minute areas of necrosis caused by emboli of endothelial cells occluding capillaries. Nothing has been found to indicate that minute necroses are more frequent in the cases

with endothelial separation than in those without. The absence of minute necroses seems to justify the classification of those instances in which the blood-vessel was entirely filled with endothelial cells as separation of the third degree rather than to consider them conditions of embolism of endothelial cells.

Moreover, if the condition were an intra-vital one the vessels would be expected to show certain reparative changes, especially in the cases of the large separations of the first and second degrees, and deposits of fibrin upon the wall of the vessel might be looked for. No such changes were observed in this series.

It has been said that the process would seem a vital one because the desquamated portions are intimately mixed with blood corpuscles. But the blood may remain fluid within the vessels for a comparatively long time. Desquamation might take place during this period and still allow the cells to lie among the corpuscles; the intimate mingling of the cells with the blood corpuscles might perhaps be brought about by amœboid movement of the desquamated cells to which Mallory [3] has called special attention in connection with the proliferation of endothelial cells caused by toxines. The question of a possible relationship between toxins and endothelial detachment, due to infection during life or to post-mortem bacterial invasion of the tissues, is an interesting one, but the facts at hand hardly justify its discussion at this time. We know practically nothing of the length of survival of endothelial cells after general death has taken place. The morphological observations recorded in this study are hardly exact enough to determine whether or not the detached endothelial cells had exercised their power of amœboid movement.

Many desquamated cells lie next the wall with no blood corpuscles between them and the vessel wall, desquamation having taken place probably after the coagulation of the blood.

From the findings in this series it would appear that endothelial separation, while in some cases possibly the result of artefact, in many cases cannot be so considered, and in these cases it is probably not a vital process because no evidence of

[3] Loc. cit.

cellular emboli or of reparative changes is present. Neither is it a late post-mortem change since the cells are in many cases embedded among the blood corpuscles. It might occur during the death agony, perhaps produced by irregular contractions in the arterioles similar to those occurring in the heart at that time. But the fact that the condition is found also in the veins might make this seem unlikely.

No doubt observations upon suitable material other than from post-mortems—from surgical operations and similar sources—would shed much light, not only upon the question of the time of production of endothelial separation, but also upon the question of its causes.

EXPLANATION OF PLATES.

PLATE IX.

Fig. 1.—Showing endothelial separation of the 1st degree in a central vein in a congested liver.

Fig. 2.—Showing endothelial separation of the 2d degree in an artery in a fibrous kidney.

PLATE X.

Fig. 3.—Shows endothelial separation of the 3d degree in a somewhat hyaline artery in the spleen.

Fig. 4.—Shows endothelial separation of the 4th degree in a thickened artery in the pancreas.

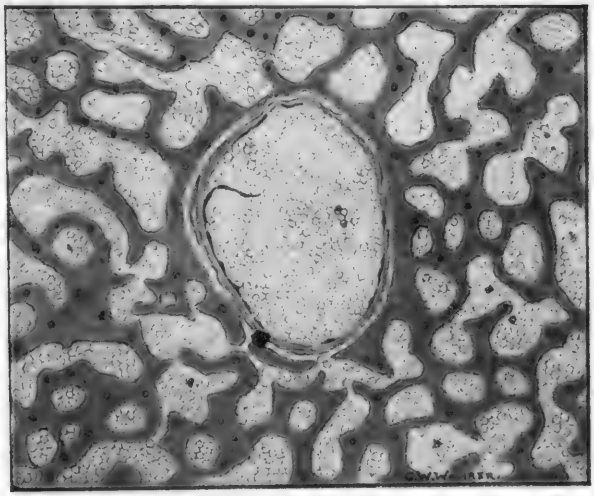


FIG. 1.

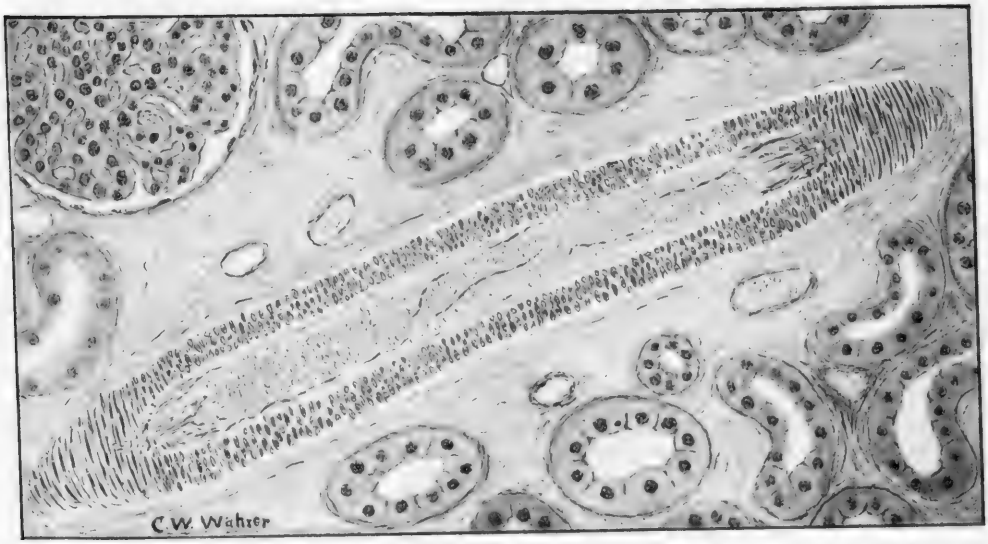


FIG. 2.

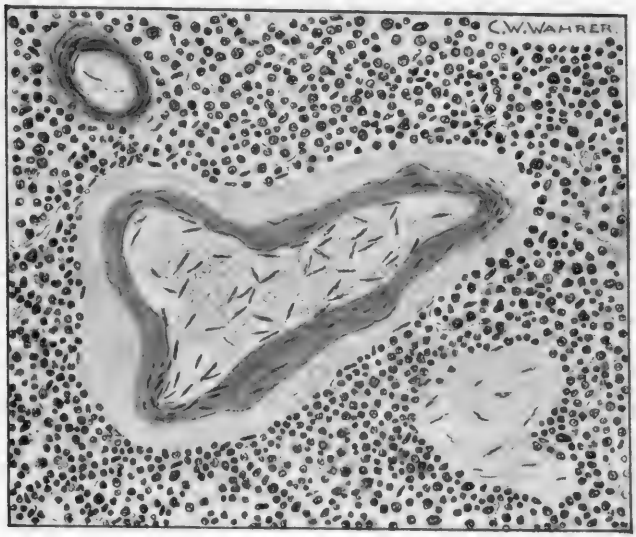


FIG. 3.

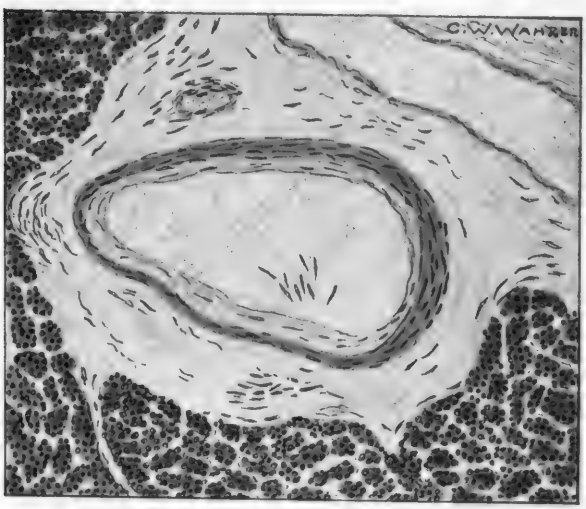


FIG. 4.

A STUDY OF THE PROTECTIVE ACTION OF SNAKE VENOM UPON BLOOD CORPUSCLES.

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(*From the Rockefeller Institute for Medical Research, New York.*)

The present article deals with certain new facts concerning the action of venom upon blood. For the purpose of defining the scope of this study, it will be advisable to review briefly the more important contributions to our knowledge of the action of snake venom upon the blood.

The hitherto well-established effects of venom upon the blood are the hæmolytic and hæmagglutinative action upon the corpuscles, and the inhibitory and accelerating action upon the coagulation of the plasma. The reducing action upon oxyhæmoglobin has also been recognized. It was Mitchell and Reichert,[1] probably, who first definitely described the phenomena which are now known as hæmolysis and agglutination, and these same investigators emphatically called attention to the loss of coagulability of the venomized blood. Martin and Smith,[2] Phisalix,[3] Kanthack,[4] Mitchell and Stewart,[5] and Stephens and Myers [6] pursued the subject further and, while confirming the studies of Mitchell and Reichert, succeeded, at the same time, in extending our knowledge of venom activity. Martin,[7] indeed, found that large doses of the venom of a certain Australian snake produce extensive intravascular clotting, a fact which Lamb [8] also made out for the venom of an Indian viper, *Daboia russelli;* and Flexner and Noguchi [9] pointed out that the blood clots which are found in the right side of the heart and the pulmonary artery after the intravenous infusion of daboia venom contained agglutinated red blood corpuscles. It was Lamb[10] who first demonstrated the power of venom to cause coagulation of citrated blood *in vitro.* Cunningham [11] observed the discolorizing effect of venom upon blood

corpuscles, from which he erroneously concluded that venom exerts its chief toxic action upon blood, and the various phenomena commonly observed in poisoned animals are merely the consequences of the loss of the respiratory function by the altered red corpuscles. Flexner and Noguchi [12] found the mechanism of venom hæmolysis to be analogous to serum hæmolysis, and demonstrated the occurrence in venom of hæmolytic amboceptors in Ehrlich's sense. Calmette [13] discovered the important fact, destined to have very far-reaching consequences, that one of the venom activating principles is thermostable, the other being of the nature of the usual serum complements. This observation of Calmette led immediately to the brilliant discovery of lecithin-venom activation by Kyes,[14] who also succeeded in preparing a crystalline compound of venom hæmolysin and lecithin which possesses all the native hæmolytic strength of the venom. The independence of the hæmatoxic and other toxic principles, the number of which is surprisingly large, has been shown by the later studies of Flexner and Noguchi.[15]

The properties of venom already enumerated depend on principles which, with the possible exception of the hæmolysin in the lecithin compound, have not been separated in a state approaching purity. Mitchell and Stewart [16] described another property of crotalus venom which had apparently escaped the attention of previous investigators. They observed that in a mixture of blood and fresh venom, in equal parts, the corpuscles, instead of undergoing hæmolysis, were actually preserved from disintegration for a period considerably greater than in the control specimens to which no venom had been added. On the other hand, if the amount of venom employed was less than 10 per cent of the mixture, hæmolysis occurred in the usual way. Mitchell and Stewart were led by their experiments to regard the once dried venom as being a less effective preservative than the fresh secretion, and they noted that, of the corpuscles tested, those of the rattlesnake were most perfectly protected by crotalus venom. Stephens and Myers [17] described a similar phenomenon in the case of cobra venom and human blood, although they do not state how long the corpuscles can be preserved in this way. Flexner

and Noguchi [18] in their first studies noted a protection of rabbit corpuscles by means of rattlesnake venom, and, somewhat later, Kyes [19] and Kyes and Sachs [20] encountered the same phenomenon in the course of their studies with cobra venom, and Lamb [29] with daboia venom.

While the earlier investigators merely recorded their results, Kyes and Sachs [21] endeavored to explain the phenomenon. They accounted for it by supposing a complement (lecithin) deviation or "side-tracking" through the action of the large excess of venom hæmolytic amboceptors which were present in the mixture of blood and venom. Their crucial experiments were conducted as follows: Blood corpuscles were treated with a large excess of cobra venom and thoroughly washed to remove the excess of venom. Since, as Flexner and Noguchi [22] had shown, the venom amboceptors became attached to the corpuscles, the latter could now be hæmolyzed by contact with suitable complements (serum, lecithin). In the control experiments the excess of venom was not removed by washing, and in them the tubes containing the larger amounts of venom either failed entirely to show hæmolysis after addition of complement, or the amount of hæmolysis was greatly diminished.

In the course of a recent study of the influence of venom in promoting the hæmolysis caused by physical and chemical agents, this protective action of strong solutions of venom was given special attention, and the present communication deals with the results of this investigation. It may now be regarded as well established that when the optimum of the hæmolytic action of venom is exceeded, the degree of hæmolysis which it is capable of producing diminishes progressively as the dosage of the venom increases. Among the natural biological hæmolysins, venom alone is known to possess this property, but, in the course of a study of bacteriolysis by certain immune sera of high potency, Neisser and Wechsberg [23] observed an inhibition of bactericidal effects when an excess of amboceptors, relative to the complement content, was brought into the bacterial suspension. A similar although probably a distinct phenomenon has been described by Detre and Sellei [24] in their studies of hæmolysis caused by bichloride of

mercury. But excepting the few experiments made by Kyes and Sachs, upon which they base their notion of complement deviation, no really extensive study of this property of venom has been made.

The explanation offered by Kyes and Sachs involves the belief that the principle, which is responsible for the protection, is identical with that which causes, under favorable conditions, dissolution of the corpuscles. If it can, therefore, be shown that it is not the hæmolysin which is protective, but some other principle, the notion that lecithin deviation explains the appearances can not be maintained. It is possible by means of suitably arranged experiments to test fully the assumption of a protective action exerted by venom and due to hæmolysin, and, further, to study other features of this action with a view of acquiring a more complete understanding of it.

The experiments to be related were devised to determine whether the protection is effective only against complements, or whether it operates as well against other hæmolytic agencies from which the biological factors are entirely eliminated. These several aspects of the subject will be dealt with in the following pages.

Preliminary Remarks upon the Action of Venom upon Blood Corpuscles.—In the experiments to be given, the blood of the horse was chiefly used, and it is meant unless another kind of blood is specified. Unwashed horse corpuscles are fairly susceptible to venom hæmolysis, while the corpuscles after washing (three washings in fifteen or twenty times their volume of salt solution at each operation) are insusceptible to venom. The horse has the advantage of yielding small or moderate quantities of blood at not too frequent intervals without causing perceptible changes in the resistance of the corpuscles. By using a single horse and establishing the resistance of its red corpuscles a standard can be obtained that, within small limits, is constant. In carrying out the experiments on resistance this condition was essential.*

* Theobald Smith (*The Journal of Medical Research*, 1904, xii, 385) has described a reduction in resistance to salt solutions of corpuscles of horses sub-

The blood corpuscles of the horse present another advantage in their greater resistance to the mechanical injury involved in defibrination and washing.

The defibrinated blood in 0.9 per cent salt solution is susceptible to cobra, crotalus, and moccasin venoms, the varieties which were employed. A mixture of blood suspension and cobra venom, in which the venom is contained in greater quantity than 5 per cent of the dry poison, will remain undissolved when kept for several hours at 20° C.; but at the end of twelve hours complete hæmolysis will have taken place.

In general it may be stated that rapidity and completeness of hæmolysis depend upon the proportional amounts of venom and blood in the mixtures: the greater the quantity of venom and the smaller the amount of blood, the slower and less perfect the hæmolysis. There is, however, one factor which controls to a large extent the rapidity and degree of hæmolysis, namely, the presence of serum constituents in the mixture. So long as a trace of serum remains, complete dissolution can not be prevented by any practicable concentration of venom. An equal mixture of defibrinated blood and 25 per cent cobra venom* solution will undergo hæmolysis in one or two hours.

It has already been stated that the thoroughly washed horse corpuscles are not hæmolyzed by venom no matter what the degree of concentration. If cobra venom be used in strengths above 4 per cent with a corpuscular suspension of 5 per cent, no change may take place in the mixture in many weeks; while with a quantity of venom as small as 0.1 per cent the corpuscles will disintegrate rather more quickly than in the control tubes which are not entirely sterile.

Corpuscles which had been brought into contact with the stronger solutions of venom were tested for resistance to salt solutions of varying tonicity. These tests disclosed the unexpected fact that corpuscles thus venomized, and in the

jected to repeated bleedings in the production of diphtheria antitoxin. Our practice was to take relatively small quantities of blood at intervals of a number of days from a strong horse that had not previously been used for any experiment.

*The percentage of dried venom is always meant.

presence of an excess of venom, are not hæmolyzable even by water.

At the same time their susceptibility to heat is changed. It has been found that control tubes of blood corpuscles alone are hæmolyzed completely in from one hundred and seventy-five to one hundred and eighty minutes when kept at the constant temperature of 53° C. In the presence of venom of a concentration not exceeding 1 per cent, complete hæmolysis will take place at this temperature in from five to fifteen minutes; with a concentration of venom as low as 0.01 per cent thirty minutes will be required; with a concentration of 10 per cent there is no perceptible change in the corpuscles for the first twenty minutes, after which laking commences. This last laking is not, however, typical. A bright hæmoglobin color does not appear in the fluid, but the cells undergo disintegration, the color of the mixture becomes coffee-like, and in about one hour a turbid precipitate of cell particles and venom granules can be made out under the microscope.

Strong solutions of venom are capable of protecting the corpuscles from destruction by water, but venom solutions below 2 per cent in strength render the corpuscles more sensitive to salt solutions, as measured by the tonicity of the fluid for corpuscles; and as the strength of the venom falls from this limit the susceptibility, as measured by the degree of tonicity, diminishes.

Is the Protective Action Due to Venom Hæmolysin?—The first question to be considered is whether the protective action of strong solutions of venom is in any way dependent on the hæmolytic amboceptors. Since this protection is exhibited against water and heat, it is safe to assume that it bears no necessary relation to complement deviation. However, even these reactions do not prove that the hæmolysin is unconcerned in the process. Some other explanation, one, namely, in which an alteration in solubility of the protoplasmic constituents of the cells is concerned, has more likelihood of fitting the facts observed, but nevertheless it is necessary to exclude by suitable means any possible participation of the hæmolysin.

There is a marked difference between the effects produced by complements present, in an available form, before the venom has acted on the corpuscles and those produced by complements added after the treatment with venom. Should even a trifling quantity of serum be left with the corpuscles, it is not practicable to obtain the protective effect of venom, while if the effect is once produced and the excess of venom allowed to remain, very large amounts of serum or of lecithin fail to bring about hæmolysis.

The first set of experiments relates to the difference in effect produced by two concentrations of venom. In each experiment the washed corpuscles were in 5 per cent suspension, and were allowed to remain in contact with the venom for two hours before any complement was added. The mixtures were all made in 0.9 per cent solution of salt. The two strengths of venom were 10 and 2 per cent. Table I expresses briefly the result.

TABLE I

5 per cent horse corpuscles.	Amount of corpuscular suspension. c.c.	Amount of lecithin and fresh serum. c.c.	Result, at 20° C.
10 % Cobra venom	0.3	+ 0.9 % salt sol. 0.7	= No hæmolysis
	"	+ $\frac{1}{1000}$ N. lecithin 0.7	= No hæmolysis
	"	+ $\frac{1}{100}$ N. " 0.7	= Doubtful result, owing to the formation of granules, but no hæmolysis after 6 hours.
	"	+ horse serum 0.7	= No hæmolysis; strong discoloration after some hours.
2 % Cobra venom	0.3	+ 0.9% salt sol. 0.7	= No hæmolysis.
	"	+ $\frac{1}{1000}$ N. lecithin 0.7	= Complete hæmolysis in 5 minutes.
	"	+ horse serum 0.7	= Complete hæmolysis in 2 hours.
	"	+ horse serum 0.07	= Complete hæmolysis in 4 hours.
Control 0.9 % Salt sol.; no venom	0.3	+ 0.9 % salt sol. 0.7	= No hæmolysis.
	"	+ $\frac{1}{1000}$ N. lecithin 0.7	= Complete hæmolysis in 8 hours.
	"	+ $\frac{1}{100}$ N. " 0.7	= Complete hæmolysis in 1 hour.
	"	+ horse serum 0.7	= No hæmolysis.

Examination of the table shows that the corpuscles which are acted on by 10 per cent venom are not hæmolyzable by quantities of fresh horse serum and lecithin, which far surpass the amounts theoretically required to bring about solution; while with the venom in 2 per cent solution the corpuscles were perfectly dissolved by lecithin and serum. The quantity of complements used in the experiments given in the table was in great excess, and the excess in the tests with 10 per cent venom was ten times greater than in the tests with 2 per cent venom, and one hundred times greater than actually required under optimal conditions.

Before discussing the participation of the venom-hæmolysin in the protective action, it will be necessary to describe the influence of heat upon the reaction. As a preliminary step, the influence of high temperatures upon the hæmolytic action of cobra venom was made. It was found that the filtrate from the coagulum of venom heated for five minutes at 100° C. was still hæmolytic, and, indeed, quantitatively in the same degree as the unheated venom.

Tubes containing a 25 per cent solution of cobra venom in 0.9 per cent salt solution were heated to 95°, 100°, and 135° C., respectively. The temperatures of the first two tubes were maintained for five minutes, while the last tube was kept at the high temperature in the autoclave for one hour. When venom in solution is heated to 95° C. it becomes milky in appearance, while at 100° C. coarse particles of coagulated matter separate. At the temperature of 135° C. the coagulum shrinks to a solid, spongy mass, leaving the fluid portion clear. The heated venom was now tested and it was found that the specimen heated to 95° C. was practically unaltered in its hæmolytic and protective action; while at 100° C. it had lost a portion of its effect only, and at 135° C. it had lost entirely both of these properties.

The next series of tests was made with the separated portions, coagulum and filtrate. For this purpose the specimen heated to 95° C. was employed, and to obtain a perfectly clear fluid, filtration through a Berkefeld bougie was resorted to. The results of this test were conclusive; the filtrate had no protective action,

while the coagulum when suspended again was protective. The filtrate from the specimen heated to 100° C. was also non-protective. The filtrates from both of these fractions contain the hæmolytic principle in undiminished amounts.

These experiments prove definitely that the protective principle is distinct from the hæmolytic amboceptors, and it is interesting to note that the coagulum from the specimen heated to 95° C. is still capable of exerting protective influence upon blood corpuscles. It seems safe to state that the coagulated proteid is not the protecting body; and hence it is a fair assumption that in process of coagulation the protective principle is mechanically carried down under conditions of union which still permit its entering into combination with blood cells.*

Although the filtrate from a solution of venom heated to 95° or 100° C. has no protective action, such a filtrate exerts an injurious action on the corpuscles, whereby they are rendered more easily hæmolyzed by salt solution. The washed corpuscles of the horse are laked by 0.44 per cent salt solution, but after treatment with the filtrates mentioned they are instantly laked by solutions of 0.5 per cent. The temperature of 135° C. destroys not only the protective but also the injurious body; and at that temperature the filtrate is without hæmolytic action even in the presence of suitable complements.

Our experiments lead us, therefore, to regard corpuscular protection by strong solutions of venom as a peculiar effect which is opposed to hæmolytic action, and one which has thus far received neither adequate attention nor probable explanation. Moreover, it would seem that the hæmolyzing principle in venom is directly injurious to corpuscles even in cases in which suitable complements are absent. Until a special injurious agent which predisposes corpuscles to laking by physical agents is discovered in venom, we are inclined to believe that the injurious action of the filtrates is due to venom hæmolysin.

* In view of the fact that in his experiments which led to the view of complement deviation by venom amboceptors Kyes employed rabbit corpuscles, I have made a series of tests with these cells. My results show that rabbit corpuscles are somewhat less perfectly protected than some other cells, but

A passing consideration should be given to venom agglutinins, since it is at least conceivable that the agglutinated masses of corpuscles arising from venom treatment are less readily affected by laking agents than the separated corpuscles. But since the

they exhibit unmistakable evidences of protection to heat, saponin, and water. Moreover, they also show the paradoxical reaction to salt solution, since the super-venomized corpuscles show at one point a greatly lessened resistance to solutions of sodium chloride. A table is appended in which these results may be observed.

Amount of 5 % cobra venom solution c. c.	1 c. c. of 5 % suspension of rabbit blood (washed)		
	Blood No. I.		Blood No. II.
	Temperature of 53° C.	1 % saponin sol.	Reaction to water and NaCl solution.
1	No hæmolysis, precipitate formed, cells disintegrated in two hours.	Slight hæmolysis	Slight hæmolysis in water.
0.5	No hæmolysis, precipitate formed, cells disintegrated in two hours.	" "	Partial hæmolysis in water.
0.25	Complete hæmolysis in 9 minutes	Partial "	Complete hæmolysis in 0.5 % salt sol.
0.1	" " 5 "	Complete "	" " 0.53 % " "
0.075	" " 3½ "	" "	" " 0.68 % " "
0.05	" " 2 "	" "	" " 0.566 % " "
0.025	" " 2½ "	" "	" " 0.55 % " "
0.01	" " 9 "	" "	" " 0.535 % " "
0.005	" " 17½ "	" "	" " 0.52 % " "
0.001	" " 35 "	" "	" " 0.48 % " "
0.0005	" " 42 "	" "	" " 0.458 % " "
Control (no venom)	" " 130 "	" "	" " 0.43 % " "

In repeating Kyes's experiments I found that the points of protection coincided with the points of so-called deviation of complement as is shown in the next table.

Amount of 5 % cobra venom solution c. c.	1 c. c. of 5 % rabbit blood (defibrinated)		
	Blood No. I.	Blood No. II.	Blood No. III.
1	No hæmolysis	No hæmolysis	No hæmolysis
0.5	" "	Trace "	" "
0.25	Trace "	Marked "	Slight "
0.1	Moderate hæmolysis	" "	Moderate hæmolysis
0.075	Almost complete hæmolysis	Complete hæmolysis	Complete "
0.05	Complete hæmolysis	" "	" "
0.025	" "	" "	Almost complete hæmolysis
0.01	" "	Almost complete hæmolysis	" "
0.005	" "	" "	Marked hæmolysis
0.001	Almost complete hæmolysis	Much hæmolysis	" "
0.0005	Trace hæmolysis	No "	No "
Control (no venom)	No "	" "	" "

venom agglutinins are destroyed at relatively low temperatures their participations can be quickly eliminated. I have also tested abrin and ricin, which are strongly hæmagglutinative, without finding a corresponding protection. Indeed, corpuscles treated with abrin are slightly reduced in resistance, while corpuscles treated with ricin show about the normal reaction to salt solution.

The serum of the rattlesnake is highly agglutinative and hæmolytic for corpuscles of the horse, and yet it does not protect them in any degree. The corpuscles, after a contact of twelve hours with the inactivated rattlesnake serum in excess, were hæmolyzed by salt solution of 0.45 per cent strength.

That other coagulated proteids and mucin do not contain any such protecting principle is shown by the negative results obtained with coagulated serum of the horse and of the rattlesnake, and with salivary mucin.

The Effect of Washing the Corpuscles after Venomization.—Attention has been drawn to the fact that the property of venom which is being described is operative only in the presence of an excess of venom. Corpuscles once protected can be diluted with water indefinitely without any laking taking place, so long as the excess of venom is not removed by washing, and other evidences of protection, such as increased resistance of the corpuscles to heat and to various chemical laking agents, can be demonstrated. If, however, the venomized corpuscles are subjected to repeated and thorough washing with 0.9 per cent salt solution, it is found that the protection disappears and, moreover, the corpuscles are now more easily laked than normal blood cells.

Four mixtures of washed corpuscles and venom were prepared. The corpuscles were used in 3 per cent suspension in salt solution, and the venom strengths were 10, 1, 0.1, and 0.01 per cent. The period of contact was six hours. A portion of each mixture was removed and washed in 0.9 per cent saline four times, twenty times the volume being used at each washing. Two sets of tubes, after dilution four times with saline solution, were exposed to the temperature of 53° C. and the results noted. They are reproduced in Table II.

TABLE II

Strength of venom solution.	Washed venomized corpuscles.	Unwashed venomized corpuscles.
10 %	Complete hæmolysis in 20 minutes	No typical laking, but general disintegration of cells in 60 minutes; the fluid presented a light brownish hue.
1 %	Complete hæmolysis in 25 minutes	Complete hæmolysis in 6 minutes.
0.1 %	Complete hæmolysis in 25 minutes	Complete hæmolysis in 12 minutes.
0.01 %	Complete hæmolysis in 30 minutes	Complete hæmolysis in 30 minutes.
Control (no venom)	Complete hæmolysis in 170 minutes	Complete hæmolysis in 175 minutes.

Table II shows that horse corpuscles suspended in the venom solutions of strengths above 0.1 per cent absorb about the same quantity of venom irrespective of the actual concentration of the solutions. The quantity of venom absorbed sufficed to bring about laking in twenty-five minutes, the mixtures having been kept during this period at 53° C.; and this amount of venom is apparently the maximum quantity which the corpuscles are able to retain after repeated washings with the saline solution.

There is no method of ascertaining with absolute accuracy whether this quantity was originally absorbed, or whether it is the part of the original quantity fixed to cells and retained with great avidity. An approximate estimation could, of course, be made of the amount of venom remaining in the fluid part of the mixture after simple contact of the blood-cells and venom; but since, in carrying out this estimation, no account can be taken of the venom which is loosely and mechanically held by the corpuscles, figures approaching accuracy are impossible.

That venom directly injures blood corpuscles has been mentioned, but Table II is valuable in showing that this injury can be expressed in quite definite figures. I know of no series of experiments in which laking by means of heat has been used to

determine the degree of decreased resistance of blood cells. The method seems to promise a fair degree of accuracy, and it also would seem to promise the discovery of substances of primary injurious nature, which until now have been assumed to be inactive except in the presence of suitable complements.

In order that this primary injurious action may be expressed, the concentration of the venom solution must be kept between 0.1 and 1 per cent. Higher concentrations either begin to be protective or do not increase the injury, because they are not so entirely absorbed. In any case an excess of venom would appear not to be present in the unheated mixtures, for hastening of heat hæmolysis is not observed in the venomized corpuscles which have previously been washed. Hence the question arises whether at the high temperature more venom is not absorbed than at 20° C.; from this increased fixation and injury perhaps the greater susceptibility to laking proceeds. No answer to this question is attempted at present; but I shall now proceed to the next series of experiments which are concerned with the effect of repeated washings in saline solution upon the tonicity of the venomized corpuscles, as measured by their resistance to salt solutions of different strengths.

TABLE III

Strength of venom solution.	Venom removed.	Venom not removed.
10 %	0.51 %	Not hæmolyzable in water.
1 %	0.49 %	0.48 %
0.1 %	0.48 %	0.465 %
0.01 %	0.465 %	0.44 %
Control (no venom)	0.44 %	0.44 %

The striking difference in the manner of reaction to hypotonic solutions between the corpuscles acted upon by strong venom solutions and by solutions of weaker concentration is well illustrated in this table. After the action of 10 per cent venom, the corpuscles can be diluted indefinitely with water without showing any tendency to dissolve, while the same corpuscles, after

repeated washing with 0.9 per cent salt solution, show a higher hypotonic index than normal—0.51 per cent as compared with 0.44 per cent. The significance of this behavior is quite plain. Strong venom solutions react in some manner with the corpuscles so that they are rendered insoluble in water; the insoluble compound is, however, capable of being broken up by certain chemicals—in this case sodium chloride. But whether it is the sodium chloride as such, or the free ions in the mixture, the experiment does not show. It may be remarked here that the protective property is not quickly removed by the saline solutions, but several washings are required to remove it. It would seem, therefore, that an excess of sodium chloride alone does not suffice, but some other factor is concerned.

Since it has now been shown that the entire protective effect of the venom, as regards water hæmolysis, can be removed by washing the corpuscles with the saline solution, the question arises whether this process restores the susceptibility of the corpuscles to lecithin and serum hæmolysis. It has already been stated that the presence of serum in the mixture will prevent the strongest solutions of venom from causing protection; and now it may be said that the protection once removed by salt solution the corpuscles become susceptible to serum and lecithin hæmolysis.

The next table gives the results obtained with lecithin and horse serum. The conditions of the experiment were similar to those of the preceding experiments. The period of contact of venom and washed corpuscles was two hours; and the washing was always done with 0.9 per cent salt solution. The volume of suspended corpuscles was 3 cubic centimeters, to which 0.5 cubic centimeter of $\frac{1}{1000}$ N. lecithin or 1 cubic centimeter of horse serum was added. The observations in the case of the horse serum were made at the end of four hours.

It appears that washing with salt solution exposes the protected corpuscles to laking with serum and lecithin. This susceptibility to laking is due to the fixation of venom hæmolysin by the corpuscles. When the activity of very strong solutions, after removal of all unattached venom, is compared with the

activity of weak solutions, in which subsequent washing is omitted, a marked difference in the rapidity with which hæmolysis occurs is apparent. The difference may depend, possibly on one of two causes, first, that the corpuscles in the absence of complement actually fix an insufficient quantity of hæmolysin to produce rapid laking; or, next, the washing removes a part of the hæmolytic amboceptors already attached.

The Action of Certain Substances upon the Venomized Blood Corpuscles.—The effects of water, salt solution of different concentrations, and heat upon the venomized corpuscles have been

TABLE IV

Strength of venom solution.	Lecithin.		Horse serum.	
	Venom removed.	Venom not removed.	Venom removed.	Venom not removed.
10 %	Complete hæmolysis in 28 minutes	No hæmolysis	Complete hæmolysis	No hæmolysis.
1 %	Complete hæmolysis in 28 minutes.	Complete hæmolysis in 7 minutes	" "	Complete hæmolysis.
0.1 %	Complete hæmolysis in 29 minutes.	Complete hæmolysis in 9 minutes.	Partial hæmolysis	" "
0.01 %	Complete hæmolysis in 30 minutes.	Complete hæmolysis in 28 minutes.	" "	" "
Control (no venom)	Complete hæmolysis in 8 hours.		No hæmolysis	

recorded. The next series of experiments deals with the action of still other definite agents, such as acids, alkalies, etc., upon the protected cells. The method of procedure was as follows: Four tubes, containing 4 cubic centimeters each of 5 per cent cobra venom in 0.9 per cent salt solution and 6 per cent of washed horse corpuscles, were allowed to stand for two hours at 20° C. Tube No. 1 received 4 cubic centimeters of water, and tube No. 2 received 20 cubic centimeters of water; the corpuscles were permitted to subside when the clear fluid was decanted and the volume of the residue brought up to 8 cubic centimeters. Tube No. 3 received an additional 4 cubic centimeters of salt solution, while the residue in tube No. 4 was washed once in 20 cubic centimeters of salt solution and suspended in 8 cubic centimeters of saline solution. The actual tests were made by measuring 2

cubic centimeters of each mixture into a test-tube, to which the solutions of chemicals were added gradually until an amount was introduced which produced instantaneous hæmolysis. The chemicals were dissolved either in water or in saline solution, according as they were to be added to blood cells diluted in one or the other of these fluids. The results are given in the next table.

TABLE V

Venomized corpuscles.	$\frac{1}{50}$ N. HCl c. c.	$\frac{1}{50}$ N.NaOH c. c.	$\frac{1}{50}$ N. NH_3 c. c.	0.1 % saponin. c. c.
1. Dilution with water	0.125	0.75	1.75	No hæmolysis.
2. Sedimented and resuspended in water	0.12	0.7	1.	" "
3. Dilution with saline solution	0.33	1.5	2.	" "
4. Washed with and resuspended in saline solution	0.25	0.8	0.3	Complete hæmolysis, 0.35
5. Control, no venom	0.5	1.75	0.4	" " 0.45

An analysis of this table shows an essential agreement between the manner of action of hydrochloric acid and sodium hydroxide. These substances cause hæmolysis in smaller quantities when added to the watery suspensions than with suspensions of corpuscles in saline solution, the difference being approximately as 1 : 2 or 1 : 2.5. On the other hand, after removal of the excess of venom by washing in the saline solution, the corpuscles show a greatly reduced resistance, since they are about twice as readily laked by these chemicals as the control without venom. On the other hand, in the watery medium the super-venomized corpuscles are about four times as susceptible to hydrochloric acid and twice as susceptible to sodium hydrate as the control specimens. This apparent paradox is probably explained by the nature of the medium, for, the insoluble compound of venom and proteid once dissolved, the previously protected corpuscles

immediately become the victims of water-laking. The reduction of resistance exhibited by the venomized cells suspended in salt solution is an expression of the injury inflicted by the venom directly upon the corpuscles in the manner already described.

On the other hand, ammonia permits unmistakable evidences of protection to be exhibited. In the first place, in the presence of excess of venom, ammonia proved to be relatively a weak solvent for the protecting compound, and, in the next place, in the case of the washed venomized cells the susceptibility of the corpuscles to the solvent action of the ammonia is increased beyond the control, although this difference is again less marked than with hydrochloric acid and sodium hydrate.

Another remarkable fact is the inability of saponin to cause laking of the protected corpuscles. According to Kobert,[25] saponin is hæmolytic probably because of its property of dissolving lecithin, which it withdraws from the corpuscles with the effect of destroying the integrity of the cells. It would seem, therefore, that the protection of the corpuscles against saponin hæmolysis might be associated with a change in the availability of the intracellular lecithin. It is improbable that venom extracts the lecithin, for if it did it is likely that the corpuscles would be directly disintegrated by the venom, but such disintegration does not occur. It is far more probable that the venom action is to bring about some physical or chemical alteration in the cells which prevents the usual effect of saponin upon the corpuscles. That, indeed, the effect of venom is not permanent, as would be the case were it to abstract the lecithin, is shown by the results of washing the protected corpuscles in the saline solution and subsequent exposure to saponin. Such washed cells are hæmolyzed by saponin even more readily than the controls. Hence the evidence is in favor of the view that venom fixation of the corpuscles is due to some change in the character of the cellular constituents, and that the venom acts also directly upon the corpuscles, producing an injury even though this injury is not at once evident. We shall return to the first aspect of this subject. The strongest solutions of venom which it is practicable

to use, like the weaker solutions, act injuriously upon blood corpuscles. The stronger solutions, however, exert a second action —a kind of fixation of the cells—which obscures the injurious action. Table VI gives the results obtained with saponin and corpuscles in the presence of an excess of venom and after its removal. The volume of the venomized blood suspension in each instance was 4 cubic centimeters. Saponin was employed in 0.1 per cent solution, which was added in increasing quantities until instantaneous hæmolysis was produced.

TABLE VI

Strength of venom solution.	Washed venomized corpuscles + 0.1 % saponin.	Unwashed venomized corpuscles + 0.1 % saponin.
10 %	0.3 c. c.	No hæmolysis *
1 %	0.35 "	0.35 c. c.
0.1 %	0.4 "	0.4 "
0.01 %	0.45 "	0.45 "
Control (no venom)	0.45 "	0.45 "

Strong venom solutions afford no protection against formic acid hæmolysis, and the protection against oleic acid is not absolute, but the degree of resistance against this acid was not wholly worked out. Ether in large quantities fails to lake the venom-protected corpuscles.

The Rapidity and the Limits of the Protective Action.—In order to determine the rapidity of the fixation of the corpuscles by venom a series of tests was made. A 10 per cent solution of cobra venom was prepared in 0.9 per cent salt solution, and to this washed horse corpuscles were added in quantity of 3 per cent. The mixture was allowed to stand at 20° C. for the period of the experiment, and at the intervals mentioned in the table portions were withdrawn and tested against salt solutions of different degrees of hypotonicity. A parallel series of tests was carried on to determine what concentration of venom in 0.9 per cent saline solution is necessary to protect the corpuscles against suspension in water. In the latter series a contact of five hours

* 2 % saponin in the mixture caused no hæmolysis after 10 days.

was employed. The following table presents the results of these experiments:

TABLE VII

DETERMINATION OF RAPIDITY OF ACTION.		DETERMINATION OF MINIMAL LIMIT OF ACTION.	
Period of contact of venom and corpuscles.	Reaction to sodium chloride solution.	Concentration of venom solution.	Reaction to sodium chloride solution.
5 minutes	Partial hæmolysis in 0.3 % sol.	2 %	Complete hæmolysis in 0.7 % sol.
10 "	Trace hæmolysis in 0.3 % sol.	3 %	Trace hæmolysis in 0.1 % sol.
20 "	No hæmolysis in 0.3 % sol.; trace in water.	4 %	No hæmolysis in water.
30 "	No hæmolysis in water.	5 %	No "
60 "	" " "	6 %	No "
		7 %	No "
		8 %	No "
		Control (no venom)	Complete hæmolysis in 0.44 % sol.

Estimation of the Protective Action of Moccasin and Crotalus Venom.—The tests under this heading were made in the manner previously given. Different strengths of the venoms were prepared in 0.9 per cent saline, and to them washed horse corpuscles were added in 3 per cent suspension. The mixtures were allowed to stand for six hours at 20° C., and were then tested against salt solutions of varying tonicity. The removal of the venom in some of the experiments was done by means of 0.9 per cent saline.

TABLE VIII

Concentrations of venom.	CROTALUS VENOM.		WATER-MOCCASIN VENOM.	
	Venom removed. Tonicity of NaCl sol.	Venom not removed. Tonicity of NaCl sol.	Venom removed Tonicity of NaCl sol.	Venom not removed. Tonicity of NaCl sol.
20 %	0.54 %	0.18 %	0.57 %	0.2 %
10 %	0.528 %	0.2 %	0.48 %	0.25 %
5 %	0.5 %	0.3 %	0.47 %	0.35 %
1 %	0.49 %	0.465 %	0.47 %	0.465 %
0.1 %	0.47 %	0.454 %	0.465 %	0.45 %
0.01 %	0.454 %	0.444 %	0.45 %	0.445 %
Control (no venom)	0.44 %		0.44 %	

The above table brings out the interesting fact that, while moccasin and rattlesnake venoms possess the property of

protecting blood corpuscles, they are in this respect much inferior to cobra venom. With neither moccasin nor rattlesnake venom was there produced absolute protection against water hæmolysis. It may be worth while to point out in this place that in ultimate hæmolytic power with defibrinated horse and dog corpuscles these venoms were about the equal of cobra venom, although crotalus venom acts more slowly and is quantitatively a little weaker than the others.* The fact of the similarity in hæmolytic power and dissimilarity in protective action is another proof of the distinction between the several principles causing the two phenomena.

TABLE IX

Period of contact before heating.	Time required for complete hæmolysis at 53° C.
Immediate	10 minutes.
10 minutes	11 "
30 "	20 "
60 "	38 "
2 hours	30 "
6 "	25 "

The Influence of Small Amounts of Venom upon Heat Hæmolysis.—In the course of the experiments a phenomenon came under my observation which will now be described. Mixtures of washed corpuscles and venom of low concentration were exposed, after varying intervals of contact, to the temperature of 53° C. At first the effect, as measured by the degree of heat hæmolysis, is small, but after a time it becomes marked. However, after a still longer interval of contact the acquired resistance undergoes diminution. A series of tests is given in Table IX. The mixture exposed to heat consisted of 20 per cent washed horse corpuscles

* For complete hæmolysis under the conditions mentioned, crotalus venom requires at 20° C. twenty-four hours and, in addition, occasional agitation of the tubes. If the comparison of hæmolysis be made after six hours and at the upper limit of dosage, the difference between cobra and moccasin venom, on the one hand, and rattlesnake venom, on the other, may be as 1 : 20, but if the reading be taken after twenty-four hours it will be as low as 2 : 3 or, at most, 2 : 4.

in 30 cubic centimeters of 0.9 per cent salt solution, and 0.2 per cent cobra venom in 1.5 cubic centimeters of the same strength salt solution. Four cubic centimeters were removed at a time at different intervals and tested.

We can see in these results the opposed action of the two properties of venom when acting together. The first effect to declare itself is apparently partial fixation of the corpuscles, but when the venom solution is too weak to accomplish this entirely, the directly injurious action of the poison makes itself felt. Hence it would seem that with such weak solutions of cobra venom as were employed in this experiment, the injurious action upon the corpuscles, in the absence of available complement, was more marked than the protective action.

The Action of Venom upon the Blood Serum, the Stroma, and the Soluble Contents of the Blood Corpuscles.—With a view of ascertaining whether venom forms with the blood constituents any difficultly soluble compound, it was tested separately upon the fluid and solid portions. Horse blood being used, the serum, the stroma, and the corpuscular contents soluble in water were tested against various venom solutions.

The stroma was prepared by laking the twice-washed, condensed corpuscles. The final product was a gelatinous mass of pale rose color, which could be suspended in water or in saline solution. The contents of the corpuscles were readily obtained by laking with water and centrifuging. They could also be diluted to any degree with water or salt solution.

The results given in the table show clearly that venom is a precipitant for certain of the constituents of the blood. The chief precipitate is obtained with the water-soluble contents of the corpuscles, although a partial reaction is also obtained with diluted serum. The stroma reacts in a minor degree with the venom, but whether or not this reaction depends on the venom agglutinin or is due to a precipitant in the real sense was not determined.

The precipitate formed with the contents of the corpuscles in pure water is dense, viscid, and finely divided, while in saline solution it is coarser and less in amount. The original color of

TABLE X

Cobra venom sol.	10 % SERUM.		10 % STROMA.		10 % CORPUSCLE-CONTENTS *		CONTROL VENOM SOLUTIONS.	
	in water.	in 0.9 % salt sol.	in water.	in 0.9 % salt sol.	in water.	in 0.9 % salt sol.	in water.	in 0.9 % salt sol.
5 %	Partial precipitation.	Cloudy, but evenly so.	Cloudy, but not more than the control.	Cloudy, but not more than the control.	Dense, viscid, and soft precipitate formed quickly. The precipitate separates out, leaving clear, slightly tinted fluid near the surface.	A coarser precipitate is formed after 18 hours. By diluting with a large volume of water a more abundant and finer precipitate appears.	Cloudy, but evenly so.	Cloudy, but not quite opaque.
1 %	A definite precipitation.	Slightly cloudy.	Slightly cloudy; the stroma settled down to the bottom.	Slightly cloudy; the stroma settled down to the bottom.	Similar to the above; but precipitate much less opaque.	A definite precipitation, but less than with water. Dilution with water caused increase of precipitate.	Slightly cloudy.	Slightly cloudy.
0.1 %	A slight cloudiness.	Almost no cloudiness.	Almost clear; the stroma sunken to the bottom.	Almost no cloudiness; the stroma is on the bottom.	More cloudy than the control, but no definite precipitate.	About same as the control.	Almost no cloudiness.	Almost no cloudiness.
No venom (control)	Almost no cloudiness.	Almost no cloudiness.	Perfectly clear; the stroma is all sedimented.	Perfectly clear; the stroma is all sedimented.	Perfectly clear.	Perfectly clear.		

* Contained hæmoglobin also.

the solution is bright red from the presence of dissolved oxyhæmoglobin, but venom quickly changes it to dark brown. The pigment is carried down with the precipitate in the watery mixture, but in the saline mixture it remains for some time in the supernatant fluid. Salt, in certain concentrations would seem, therefore, to prevent, more or less, the formation of the precipitate. Indeed, if the saline solution described is further diluted with water a more abundant precipitation takes place.

Since the protected corpuscles are quickly dissolved by acids and alkalis, the question naturally arises whether the precipitate is also easily soluble in these chemicals. It was found that acids and alkalis dissolve the precipitate in approximately the same degree as they are capable of bringing about hæmolysis of the venom-treated corpuscles.

The Action of Venom upon the Separated Constituents of Serum and Corpuscles.—Venom, as has been stated, causes a dense precipitate with an aqueous extract of washed corpuscles. When, however, sodium chloride is present in the solution in a strength equalling or exceeding 0.9 per cent, the precipitation does not take place, or occurs only imperfectly. Now, it is my belief that venom owes the protective action upon corpuscles described to its ability to form a compound insoluble in water with certain corpuscular constituents. It is, therefore, desirable to ascertain whether any particular constituent of the corpuscles is concerned in the formation of the insoluble substance, or whether all the proteids of the cells are fixed by the venom. Moreover, since salt in weak concentration is able to prevent the development of the precipitate in the corpuscular extract, it is also possible that it prevents, in serum, a similar reaction. The next experiments were made with serums in which the saline percentages had been reduced by dilution with water. The results of these tests are given in Table XI.

That simple dilution with water affects the precipitability of a compound formed by venom and serum when brought together, is shown by the results given in the table. Another way of approaching this problem is to remove the salts of the serum by dialysis. This was done for the serum of the horse and dog, and

dialysis was continued until a considerable amount of globulin had separated from the serums. It was found that only a trace of chlorides remained in the fluid. The two parts—fluid and precipitated globulin—were tested separately. The globulin which had separated belonged chiefly to the euglobulin fraction, but there still remained in the fluid parts of the serums considerable globulin precipitable by one-third saturation with ammonium sulphate. During the dialysis the volume of the serums had increased two and one-half times.

TABLE XI

	MIXTURE A.	MIXTURE B.	MIXTURE C.	MIXTURE D.
	Undiluted serum 1.0 c. c. 20 % cobra venom 0 2 c. c.	Mixture A 0.2 c. c. Water 1.0 c. c.	Serum diluted with 4 vols. of water 1.0 c.c 20% cobra venom 0.2 c. c.	Serum diluted with 4 vols. of 0.9 % salt solution 1.0 c. c. 20 % cobra venom 0.2 c. c.
Horse serum	Clear	Moderate precipitate	Much precipitate	Clear
Rabbit "	"	Moderate "	Moderate "	"
Rat "	"	Much "	Much "	"
Guinea-pig "	"	" "	" "	"
Dog "	"	" "	" "	"
Pigeon "	"	" "	" "	"
Crotalus plasma	"	Much precipitate, resembling fibrin.	" "	"

REMARKS: Controls without venom remained perfectly clear for more than 24 hours, while the controls with venom alone showed only a slight amount of precipitate in water and in 0.9 % salt solution.

The dialyzed horse serum was tested as follows: First tube—1 cubic centimeter of the fluid portion was mixed with 0.2 cubic centimeters of 20 per cent cobra venom solution; moderate precipitation. Second tube—The same mixture plus sodium chloride to 0.9 per cent strength; no precipitation. Next, the precipitate of globulin was washed in water by centrifuging and dissolved or suspended in salt solutions of the following concentrations: 0.8, 0.6, and 0.4 per cent. In the first there was perfect, in the second almost perfect, in the third imperfect solution. To the clear filtrates the 20 per cent venom solution was added, and rapid and nearly complete precipitation of the dissolved globulins occurred. When the globulin is merely suspended, as in water or weak saline, the venom quickly brings down the sus-

pended particles which, left to themselves, subside very slowly, if at all. A comparison of the amounts of sediment obtained from the fluid part of the dialyzed serum and the redissolved globulin shows that the globulin is more completely precipitable than the other constituents.

It was now decided to fraction the serum, and for the purpose the method of Freund and Joachim [26] was employed. The fractions—euglobulin, pseudoglobulin, serum albumin—were freed from ammonium sulphate by dialysis and tested with cobra venom. The most marked reaction was given by the first, the pseudoglobulin fraction giving a less reaction, and the serum-albumin fraction no precipitate. The results of the two series of tests, therefore, agree in showing the globulin to be the constituent acted upon by the venom.

The water-soluble contents of the corpuscles were also fractioned. The first fraction, yielded by one-third saturation, was proportionately large and contained a part of the coloring matter of the corpuscles. The second fraction, obtained by one-half saturation, was less in amount than the first, but contained the greater part of the oxyhæmoglobin. The third fraction, obtained by saturation, was small in quantity. The salts were removed by dialysis.

The first two fractions were deep red in color. One cubic centimeter of the solution of each was mixed with 0.2 cubic centimeters of 20 per cent cobra venom; the result was precipitation. The second fraction gave the more abundant precipitate, leaving the fluid clear of coloring matter. In order to obtain similar clearing of the first fraction additional dilution with water was required. The third fraction yielded no precipitate with the venom solution.

Since in this series of experiments the globulins and hæmoglobin were associated and it was desirable to know whether the reaction takes place between hæmoglobin and venom, as well as between the globulins and venom, separation of the hæmoglobin in a pure state was necessary.

Oxyhæmoglobin from horse corpuscles was prepared by Zinoffsky's [27] method, while that from dog's corpuscles was prepared by

laking the washed corpuscles with ether and removing the stroma in the separatory funnel. The clear solution of hæmoglobin was shaken with a large volume of ether and allowed to stand for twenty-four hours at 0° C. A large quantity of crystalline oxyhæmoglobin, which was purified by being twice recrystallized, was thus obtained.

For testing against venom the hæmoglobin was used in 0.2 to 0.1 per cent solutions in water or 0.9 per cent sodium chloride. To 1.0 cubic centimeter of the solutions of oxyhæmoglobin 0.1 cubic centimeter 20 per cent venom was added. The results were as follows: in the presence of sodium chloride no reaction occurs; in aqueous solution there is complete precipitation of the hæmoglobin of the horse, but no reaction with that of the dog. That pure hæmoglobin may react with venom is shown by these experiments; that the reaction is similar to that with the globulins in respect to solubility in salt is also shown; and that a difference in composition exists between dog's and horse's oxyhæmoglobin the experiments also indicate.

In view of these results it seemed desirable to test venom directly upon globin, which was prepared from the oxyhæmoglobin of the horse and dog by Schulz's [28] method. The solutions of globin were neutral to litmus and gave the reactions described by Schulz. To 1.0 cubic centimeter of globin solution 0.1 cubic centimeter of 20 per cent venom was added. Dog's globin gave no precipitate; horse's globin gave a voluminous precipitate which was easily soluble in acids and alkalis, but not in salt solution. Indeed, a small quantity of salt hastens the appearance of the precipitate with horse's globin.

This series of experiments would tend to show that the precipitability of oxyhæmoglobin by venom depends on the globin constituent, and that the globins of horse and dog corpuscles are distinct substances as regards, at least, their reaction with venom.

The question has already been mooted as to what the nature of the protective process described in this paper really is. That the phenomenon is independent of the hæmolytic reaction has been conclusively proven. Moreover, the immediately preceding experiments show that venom is a precipitant for several proteids

of the blood. It is doubtless upon the reaction which takes place between the venom and these precipitable proteids that the phenomenon of protection depends. One of the striking appearances of the reaction is the carrying down of the hæmoglobin by the venom; and it seems not improbable that this property might be utilized as a measure of the protection of the corpuscles, for when it is absent it is found that venom does not shield the corpuscles from water laking. The following table shows that this relation actually exists:

TABLE XII.

Origin of corpuscles.	20 % cobra-venom solution 2.0 c. c. Washed blood corpuscles 0.3 c. c.		1 % intracorpuscular contents 1.0 c. c. 20 % cobra-venom solution 0.1 c. c.	
	Behavior of the corpuscles left in the above mixture.	Behavior of the corpuscles when brought into water.	Precipitation of the hæmoglobin.	Solubility of the precipitate* in 0.9 % sod. chloride solution.
Horse	No hæmolysis	No hæmolysis	Complete	Dissolves but reprecipitates on standing.
Rabbit	" "	" "	Almost complete; fluid retains a faint color.	Partially soluble; large precipitate on standing.
Rat	" "	" "	Complete	Slightly soluble
Guinea-pig	" "	" "	None	" "
Dog	Complete hæmolysis	Complete hæmolysis	"	
Pigeon	Slight "	" "	Slight	Easily soluble.
Crotalus	No "	Incomplete "	"	" "

* The precipitate was easily soluble in different acids and alkalis.

Yet, since it is not only the hæmoglobin which is precipitated by venom, but the intracorpuscular globulins as well, it is not possible to describe the phenomenon of protection as if it depended wholly on a reaction with the former proteid. It is probable that all the precipitable proteids are acted upon and affect the result, although it seems not unlikely that the fixation of the hæmoglobin in an insoluble form is what directly prevents its diffusion out of the corpuscles into the surrounding medium.

In the course of this paper allusions to a change in the permeability of the corpuscles have been made. The question as to whether the so-called protected corpuscles are altered in their physical condition so as to exclude certain substances, which under normal conditions easily enter them, is pertinent. It is

to be considered whether the rendering of certain of the proteids insoluble is not associated with an obstruction to osmotic processes taking place between the corpuscles and the surrounding medium. If in the case of water and the protected corpuscles this fluid was successfully excluded from the corpuscles by an insoluble although superficial barrier, laking could not occur. However, many of the experiments described show that the absence of laking does not depend upon changed conditions of osmosis, but upon the formation of insoluble compounds.

A set of experiments was undertaken to determine whether, at the same time, the permeability of the corpuscles may not have been altered. For this purpose saponin was employed, since, as stated, Kobert has shown that saponin causes hæmolysis by virtue of the fact that it dissolves the lecithin of the corpuscles. Now, the failure of saponin to act upon the protected corpuscles could be due either to their impenetrability for saponin, or to the insolubility of the venom-hæmoglobin compound in the menstruum in spite of the change of the cells caused by a solution of the lecithin in saponin. The following experiment was devised with a view of determining the nature of the protection from saponin.

Twenty per cent cobra venom in salt solution was treated with washed horse corpuscles (5 cubic centimeters of venom plus 0.5 cubic centimeters of corpuscles) for three hours at the room temperature. The corpuscles were rendered water-protected and also non-hæmolyzable when treated with 2 per cent watery solution of saponin.

Two cubic centimeters of the venomized corpuscles were dropped into 150 cubic centimeters of distilled water; sedimentation was complete in twenty hours. The corpuscles were twice washed in the centrifuge with water. Examined microscopically they were entangled with venom granules. These corpuscles were protected perfectly both from water and 0.9 per cent salt solution. The sediment was mixed with an equal volume of 2 per cent solution of saponin, and the mixture was allowed to stand for ten minutes. The corpuscles were diluted and washed in water until all the saponin (determined by testing

with unprotected corpuscles) was removed. A thick suspension in water of the saponin-venomized corpuscles was now added separately to water and to 0.9 per cent saline solution (0.2 cubic centimeter of suspension to 2 cubic centimeters). The salt solution quickly caused laking, while, as was to be predicted, the water was without effect.

The interpretation of this experiment is probably as follows: The saponin enters the venom-protected corpuscles in spite of their fixation and takes up the lecithin, whereby the corpuscles are seriously damaged. However, as the venom-hæmoglobin compound is insoluble in water, no visible expression of this damage can occur. Since the venom-hæmoglobin compound is soluble in 0.9 per cent saline solution, it quickly dissolves in the latter whether it is capable of leaving the corpuscles or not. Unless the corpuscles have been previously injured, the dissolved hæmoglobin compound does not diffuse into the medium; but in view of the action of the saponin upon the corpuscles a sufficient injury has been inflicted to allow the hæmoglobin to escape. Venom protection of blood corpuscles does not seriously affect the permeability of the corpuscles, but it prevents, under many conditions, the diffusion of the hæmoglobin into the surrounding medium.

SUMMARY.

Washed blood corpuscles of certain species of animals in a concentration of about 5 per cent suspended in salt solution containing above 4 per cent of cobra venom undergo changes in their resistance to certain physical and chemical agents. They become non-hæmolyzable by water, ether, saponin, and quite strong solutions of lecithin, provided always that the excess of venom has not been entirely removed. On the other hand, certain acids and alkalis, excepting ammonia, lake the venomized corpuscles more easily than they lake normal corpuscles. Venom solutions of 2 per cent and less exert no protective property upon blood corpuscles, but they induce changes in the corpuscles whereby they are rendered more easily laked by the same physical and chemical agents.

The changes in the corpuscles upon which this protection relies can be set aside by repeated washings of the venomized corpuscles in 0.9 per cent salt solution. When the protection is removed the corpuscles are left in a state of diminished resistance to injurious agencies.

The substance in venom upon which the protective action depends is not destroyed by the temperature of 95° C., although at this temperature a part of the venom is coagulated. The protective body is found in the coagulated portion; while the venom hæmolysin is contained, in full activity, in the clear filtrate. The agglutinin for red corpuscles disappears at 75° C.; hence, the protection of corpuscles by venom depends neither upon the hæmolysin nor the agglutinin.

Blood corpuscles absorb the hæmolysin from concentrated solutions of venom as readily as from weaker solutions. This fixed hæmolysin can be brought into action, under suitable conditions, by the addition of complements. But the hæmolysin is unable, in the presence of complements in excess, to cause laking of the corpuscles unless the protection produced by the venom has first been set aside.

Cobra venom forms a precipitate with blood serum when comparatively free of salts or when diluted with water; it forms a precipitate with a watery extract of blood corpuscles, and with the separated globulins, hæmoglobin and globin of the blood. The precipitates are insoluble in water, but dissolve in weak acids and alkalis. The stroma of the red corpuscles causes no change in venom solutions.

Protection of the corpuscles against hæmolysis depends upon the formation by venom of a water-insoluble compound with certain of the corpuscular constituents, chiefly probably with hæmoglobin. There is no reason to believe that the permeability of the corpuscles is greatly altered.

The restoration of the susceptibility of the corpuscles to the ordinary laking influences is accomplished by dissolving the venom-proteid compound. Since this compound is soluble in acids and alkalis and even in a large excess of salt solution, restoration is easily effected. Corpuscles which have been pro-

tected and again rendered hæmolyzable are less resistant to injurious influences than normal corpuscles, resembling in this respect corpuscles which have been treated with relatively weaker solutions of venom. Venom, therefore, always exerts a direct injurious influence upon blood corpuscles; but strong solutions of venom may obscure this injurious effect through the predominance of the protective action which they display.

Not all blood corpuscles are susceptible to the protective action of venom, and different corpuscles display varying degrees of capacity for protection. Dog corpuscles are not protected by cobra venom, and watery extracts of dog corpuscles, as well as pure oxyhæmoglobin and globin of this animal, are not precipitated by venom solutions.

Crotalus and moccasin venoms possess this protective property, but in less degree than cobra venom.

I wish to express my gratitude to Professor Flexner for his encouragement and for many valuable suggestions during the execution of this work.

ADDENDA.

After the completion of this study the paper of Sacharoff and Sachs (*Münchener med. Wochenschrift*, 1905, lii., 297) on the hæmolytic action of photodynamic substances came to my attention. I therefore tested the protected corpuscles of the rabbit by exposing them directly to sunlight in the presence of eosin. It was found that the protected corpuscles are not subject to the hæmolytic action of this florescent substance; while the corpuscles which have had the protection removed by means of salt solution are readily hæmolyzed by eosin when exposed to the sun's rays. In keeping with the other experiments upon the injurious effects of smaller and non-protective doses of venom, it was also found that venomized but unprotected corpuscles are more readily hæmolyzed by eosin in the light than the normal corpuscles.

Another fact may be added. The supervenomized corpuscles have become insusceptible to tetanolysin.

BIBLIOGRAPHY.

1. Mitchell and Reichert.—*Smithsonian Contribution to Knowledge*, 1886, No. 647.

2. C. J. Martin and McG. Smith.—*Journal and Proceedings of Royal Society of New South Wales*, 1892, xxvi, 240.

3. Phisalix.—*Compt. rend. de la Soc. de Biologie*, 1899, 834, 865.

4. Kanthack.—*Rep. of Medical Officers of Loc. Gov. Board*, London, 1895–1896, 235.

5. Mitchell and Stewart.—*Trans. College of Physicians of Philadelphia*, 1897, xix, 105. *Memoirs of the National Academy of Sciences*, 1898, viii.

6. Stephens and Myers.—*Brit. Med. Journal*, 1898, Part I, 621. Myers.—*Journal of Pathology and Bacteriology*, 1899–1900, vi, 415. Stephens.—*Journal of Pathology and Bacteriology*, 1899–1900, vi, 273.

7. C. J. Martin.—(2).

8. George Lamb.—*Indian Medical Gazette*, 1901, xxxvi, 443.

9. Flexner.—*American Journal of the Medical Sciences*, 1903, cxxvi, 202.

10. George Lamb.—(8).

11. Cunningham.—*Scientific Memoirs by the Medical Officers of the Army of India*, 1895, Part IX, and 1898, Part XI.

12. Flexner and Noguchi.—*Journal of Experimental Medicine*, 1902, vi, 277.

13. Calmette.—*Compt. rend. d. l'Académie de Sciences*, 1902, cxxxiv, 1446.

14. Kyes.—*Berliner klin. Wochenschrift*, 1902, xxxix, 886, 918; 1903, xl, 956, 982.

15. Flexner and Noguchi.—*Journal of Pathology and Bacteriology*, 1903, viii, 379. *University of Pennsylvania Medical Bulletin*, 1903, xvi, 163.

16. Mitchell and Stewart.—(5).

17. Stephens and Myers.—(6).

18. Flexner and Noguchi.—(12).

19. Kyes.—*Berliner klin. Wochenschrift*, 1902, xxxix, 886, 918.

20. Kyes and Sachs.—*Berliner klin. Wochenschrift*, 1903, xl, 21, 57, 82.

21. Kyes and Sachs.—(20).

22. Flexner and Noguchi.—(12).

23. Neisser and Wechsberg.—*Münchener med. Wochenschrift*, 1901, xiv, 697.

24. Detre and Sellei.—*Wiener klin. Wochenschrift*, 1904, xvii, 1311.

25. Kobert.—*Beiträge zur Kenntnis der Saponinsubstanzen.* Stuttgart, 1904, 48.

26. Freund and Joachim.—*Zeitschr. f. physiologische Chemie*, 1902, xxxvi, 407.

27. Zinoffsky.—*Zeitschr. f. physiologische Chemie*, 1886, x, 16.

28. Schulz.—*Zeitschr. f. physiologische Chemie*, 1898, xxiv, 449.

29. Lamb.—*Scientific Memoirs by Officers of the Medical and Sanitary Departments of the Government of India*, 1903, No. 4, 8.

A METHOD FOR OBTAINING MASS CULTURES OF BACTERIA FOR INOCULATION AND FOR AGGLUTINATION TESTS:

WITH SPECIAL REFERENCE TO PNEUMOCOCCI AND STREPTOCOCCI.

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PLATE XI.

It is often desirable to obtain bacterial cultures in mass for chemical analysis, for purposes of inoculation, or for use in agglutination tests.

In the case of many organisms, pathogenic as well as non-pathogenic, the usual nutrient agar and broth media of our laboratories furnish, under proper temperatures and life conditions, all requisites for abundant growth, and these organisms may thus readily be obtained in mass. On the other hand, certain pathogenic bacteria grow poorly or after slight initial growth die off in the usual artificial media, and it is with much difficulty that these can be obtained in sufficient quantity for chemical study or inoculation, or in the proper conditions for studies in agglutination. This is often due either to a lack of the proper nutriment, or, if this be present, to the accumulation of products of metabolism (principally acids) which either inhibit further growth or actually bring about the death of the organisms.

Often one of the prerequisites of the abundant growth of organisms in our artificial media, either with or without free access of oxygen, is the presence of some carbohydrate or allied substance available in their metabolic processes; and all bacteriologists are familiar with the differences in amount and rapidity of growth of certain organisms in media containing available sugars as compared with the growth in the same media when such sugars are absent. When, however, the acid given rise to by the action of the organisms on the sugar reaches a certain percentage, varying in the case of different organisms, cessation of

growth occurs, and even the death of the organisms may result. With certain sensitive organisms, such as the pneumococcus, the amount of sugar often normally present in ordinary meat infusion broth is sufficient to lead to the death of the organisms after a few days, from the acid produced. On the other hand, the pneumococcus will develop only slightly or not at all in perfectly sugar-free broth.

In the study of fermentations from the time of Pasteur on, it has been known that if certain non-soluble salts, particularly calcium carbonate, not positively influencing the alkalinity of the medium, be added to solutions in which fermentations are taking place, the organic acid produced by the action of the organisms on the sugars present would unite with the calcium, liberating carbon dioxide, and be neutralized, and that so long as the other requisite nutrient materials were present in sufficient amount the organisms would continue to ferment the remaining sugar. Whether there is during this period of activity a great increase in the actual number of bacterial cells depends, however, often on other factors than the mere viability and fermentative activity of the organisms concerned, as, for instance, upon the amount of free oxygen furnished the organisms during this period, and doubtless upon other nutrient conditions not so well understood.

Although this scheme of neutralization of acids formed from the sugars normally present in ordinary media has been taken advantage of by various workers, and an addition of calcium carbonate has been recommended for the removal of acid and the preservation of the viability of organisms sensitive to even small amounts of acid [in case of the pneumococcus (*Wurtz and Mosny*); the neutralization of primary acid in the case of diphtheria broth for toxin production], no attention, so far as the writer at present knows, has been given to the use of fluid media, such as meat infusion broth, to which sugar and calcium carbonate have been added, for the purpose of getting these organisms in large amounts and in sufficiently dense emulsions to be useful for large inoculations and in agglutination experiments.

Certain experiments carried on by the writer during the last

four or five years with the above and other objects in view, not pertinent to this communication, have fully demonstrated that a massive growth may be obtained and the life and active growth of even such sensitive organisms as pneumococci and streptococci may be preserved over long periods in fluid media plus sugars, by the application of this principle of neutralization. The pneumococcus, for instance, may be preserved alive and growing at 37° C. in media rich in sugar (4 % or 5 %) from which it continuously produces acid, for long periods even extending over months. In a comparatively short time the density of the growth is astonishing to one familiar with the scanty growth of the organism in the same media under the usual conditions. It is obvious that the employment of such technique may also be useful in determining some of the factors influencing the growth or cessation of growth and factors leading to the death of organisms in certain nutrient media. Such a method may further be useful in other fields of work not germane to the present communication, but of which it is the writer's intention to deal subsequently in another paper.

The writer's chief object in making this short and outline presentation of the subject at this time is the urgency of the need of a satisfactory method of obtaining suitable cultures of sensitive and poorly growing organisms, especially pneumococci and streptococci, for purposes of inoculation and for agglutination tests.

Agglutination tests with pneumococci and streptococci, when the usual broth cultures (either with or without sugars added) or emulsions from agar are employed, have in the experience of most workers been found not only technically unsatisfactory but have given varying and often contradictory results.

On the other hand, the technique recommended by Wadsworth [1] is valuable and reliable, but when a long series of organisms, as in comparative studies, is being tested against the same or various sera, much time and energy must be consumed, when following his technique, in centrifuging and preparing the organisms for the tests.

The method here proposed, which has given eminently satis-

[1] Wadsworth, *Journal of Medical Research*, 1903, x, 228.

factory and striking results in a comparative study of pneumococci and streptococci in immune sera,—results and appearances which are entirely comparable to those familiar in typhoid, dysentery, and various other agglutinations,—is simple and particularly available for the study of pneumococci and streptococci and other organisms which ferment carbohydrates, but which thrive poorly or die out rapidly in the usual media. The streptococci often, as is well known, grow in broth, either with or without sugar, in flocculi or thickly matted masses entirely useless for agglutination purposes. By the proposed method usually a fair and often a good and satisfactory emulsion may be obtained, from which agglutinative limits may easily be determined.

The medium used should be a one or two per cent peptone broth made from meat infusion, which has been brought to neutrality before boiling and coagulation. After filtration for clearing, one per cent of dextrose (or other sugar fermentable by the given organism) and one per cent of calcium carbonate are added. If the medium be acid, the latter salt will of course bring it to neutral. The calcium carbonate may then be well distributed throughout the broth by shaking and the emulsion rapidly decanted into tubes or preferably small Erlenmeyer or Florence flasks, say 100 cubic centimeters to 150 cubic centimeters in each. These are sterilized on three consecutive days at 100° C. in the usual manner. The flasks after inoculation are placed at 37° C. and are thoroughly shaken once or twice a day to neutralize the acid formed and to break up the chains and masses in the case of streptococci. The growth may be sufficient for purposes of agglutination in two days or even in one day, but as a routine up to the present time we have employed a three or four days' growth, which seems to give more uniform results and more marked agglutination.[1] About an hour before using for agglutination tests the culture should be thoroughly shaken and the calcium carbonate and larger clumps, if present, allowed to settle during this time. The sample to be tested should then be taken from the upper portion of the fluid; or the cultures, after

[1] Such cultures may then be preserved in the ice-box. We have tested them frequently in the same sera from day to day, and have found little or no change in their limit of agglutination even after weeks.

shaking, may be centrifugated for a few minutes. This centrifugalization, however, is not necessary if one remembers that a slight primary deposit may occur which is not due to agglutination.

Such emulsions, tested in the serum of pneumonia patients and in the serum of immune animals, give clumps in every way comparable to those seen in typhoid agglutinations. (See photograph, Plate XI.)

Routine agglutination tests are made by adding one cubic centimeter of the serum dilution to one cubic centimeter of the emulsion in small test-tubes. The tubes are placed at 37° C. for two or three hours—after which time the agglutinations are often practically complete—and then transferred to the ice-box to prevent growth taking place and permit of the further deposition of the clumps of agglutinated organisms, and the final control reading made after eighteen to twenty-four hours.

For routine work on patients' sera an organism should be selected which shows little or no tendency to agglutinate spontaneously or upon the addition of normal salt solution in the control. Spontaneously agglutinating cultures of course are met with among the pneumococci, and are frequent with streptococci, but even with these the limits of agglutination can be determined with much certainty if careful comparison with the control is made.

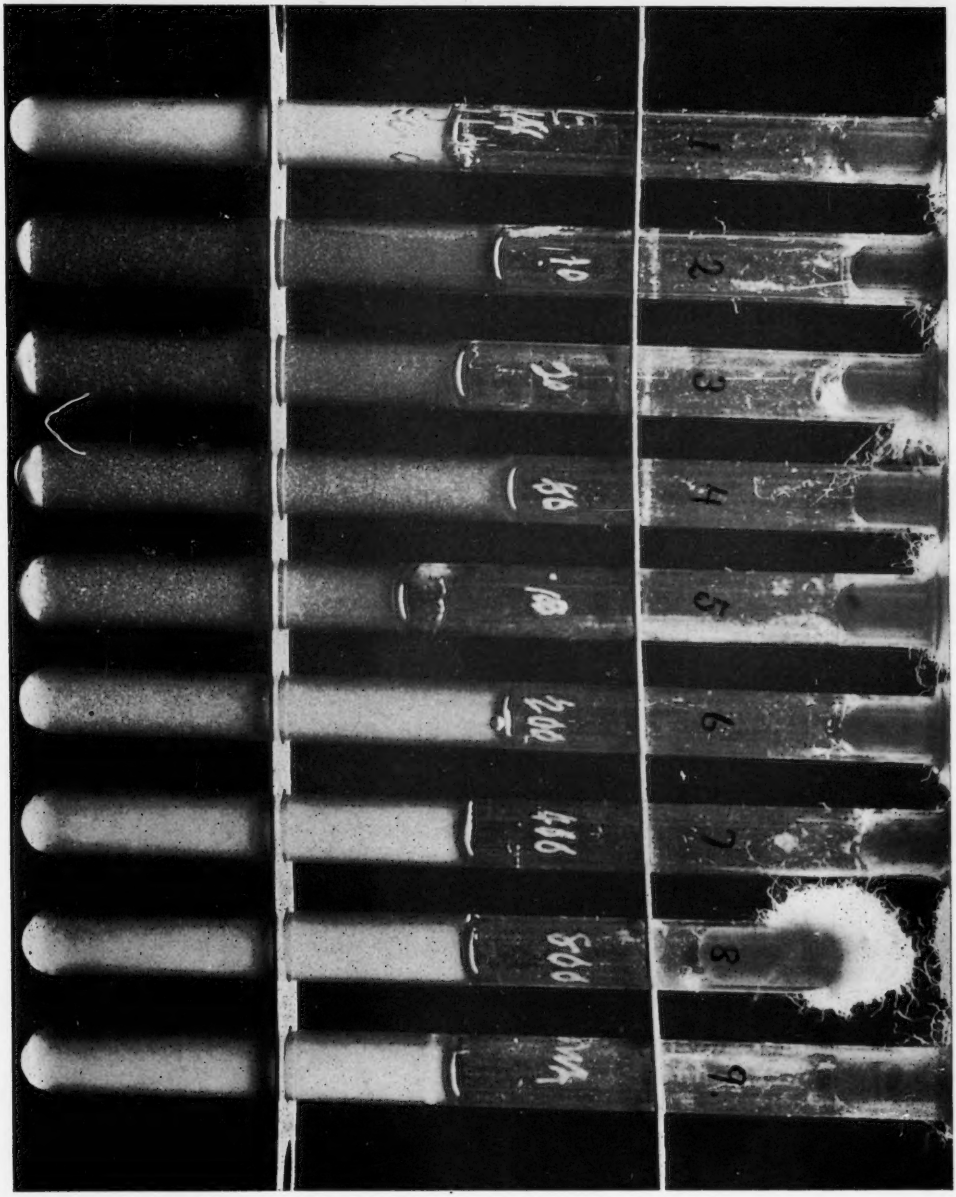
DESCRIPTION OF PHOTOGRAPH.

Plate XI.

AGGLUTINATION OF PNEUMOCOCCI, GROWN IN CALCIUM-CARBONATE-GLUCOSE BROTH, IN PNEUMOCOCCUS IMMUNE RABBIT SERUM.

The photograph represents the stage of agglutination two hours after making the mixtures, the tubes having been in the incubator at 37° C. during this time. Tube No. 1 contains a 1-20 control of the pneumococcus culture and normal rabbit serum. Tubes Nos. 2, 3, 4, 5, 6, 7, and 8 contain the pneumococcus culture and immune serum in dilutions of 1-10, 1-20, 1-50, 1-100, 1-200, 1-400, and 1-800, respectively, as indicated on the tubes. Tube No. 9 contains a control of normal salt solution and culture. The 1-10 dilution is nearly clear, and large clumps are apparent even in the photograph at 1-200. A hand-lens shows fair clumps at 1-400, and traces of agglutination at 1-800. The controls are uniformly clouded.

Each tube contains two cubic centimeters, one cubic centimeter of the culture and one of the serum dilution, which is made up with normal salt solution. The culture used in the test was grown for four days at 37° C.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